

REMARKS

The Invention

The claimed invention covers various pet foods, treats and supplements comprising a combination of lipoic acid, carnitine and coenzyme Q. This combination of compounds works effectively inside the mitochondria, as indicated in a series of three recent articles, which are not being cited as prior art but as additional data (Exhibits 1, 2 and 3). Lipoic acid is particularly suitable for the following reasons: 1) It enhances glucose uptake by increasing glucose transporters at the cell surface. 2) It has two sulfhydryl groups (unlike Vitamins C and E, which have none), which are capable of chelating iron and copper which are excessive in old age and catalyze oxidant generation reactions. 3) It is a coenzyme with mitochondrial pyruvate dehydrogenase and α -keto-glutarate dehydrogenase (unlike Vitamins A and C, which are not). 4) It is an antioxidant only in mitochondria where it is in reduced form (dihydrolipoic acid), and the reduced form is highly reactive with the oxidative species formed by metabolism. 5) It can recycle other antioxidants such as Vitamins C and E.

The combination of carnitine and lipoic acid has recently been shown to be synergistic in the following ways: 1) The combination increased both the activity and binding of carnitine acetyl transferase (CAT) (Exhibit 2, p. 1877). 2) The combination lowered brain levels of malondialdehyde (MDA), a byproduct of lipid peroxidation, which oxidizes CAT *in vitro* (Exhibit 2, p. 1879). 3) Together carnitine and lipoic acid return hepatocellular ascorbate levels in aged rats to that of young rats (Exhibit 1, p. 1872). 4) The combination increased ambulatory activity over 100% in aged rats (p. 1872). In conclusion, the combination lowers oxidant appearance and oxidative damage and reverses age-related metabolic decline (Exhibit 1, p. 1870).

All claims also recite coenzyme Q, which is a key transducer for mitochondrial oxidative phosphorylation as the rate-limiting carrier of electrons through complexes I, II and III of the mitochondrial respiratory chain. Like lipoic acid, coenzyme Q also regenerates Vitamin E and is a lipophilic anti-oxidant. Thus, all three compounds are mitochondrially active and improve mitochondrial metabolism.

Claim Objections

Claims 2, 7 and 12 were objected to because of use of the abbreviation "ALC". Applicant has amended the claims to recite the term "acetyl-L-carnitine." Therefore, this objection may be withdrawn.

Section 112, Paragraph 1 Rejection

Claims 6-10 were rejected because the Office action stated that determining the meaning of "at least one energy source" would require undue experimentation. Applicant has amended independent claim 6 by adding a Markush group of commonly understood sources of energy. Therefore, claims 6-10 no longer require undue experimentation and the rejection may be withdrawn.

Section 112, Paragraph 2 Rejection

Claims 1-2, 4-7, 9-12 and 14-15 were rejected as indefinite and unclear by reciting "a suitable antioxidant." Applicant has amended the independent claims to recite "lipoic acid", which is the suitable antioxidant meant by Applicant. The reasons for which lipoic acid is the suitable antioxidant in this invention are given above. Thus, this ground for rejection also may be withdrawn.

Section 103 Rejection

The Office Action stated that all claims were rejected under 35 USC 103(a) as being unpatentable over Sole et al. (UA 6232346 B1) in view of Ames et al. (US 5916912 A) and admitted prior arts (page 2, lines 3-22).

The Office Action noted that Sole teaches a dietary supplement for mammals comprising L-carnitine, coenzyme Q10, creatine, antioxidants (i.e., Vitamins E and vitamin C), carbohydrate, fat and protein in a formula they call their cocktail. They claim improved mitochondrial DNA transcription and translation in aged animals.

In contrast, Applicant observes that Sole states: "We consider the constituents of this path, namely Carnitine, CoQ10 and Taurine, to be the core constituents required to promote mitochondrial function. (4:43-45)" Sole goes on to state: "Three factors

namely, carnitine (critical for the transport of long chain fatty acid substrate), coenzyme Q10 (a key transducer for mitochondrial oxidative phosphorylation), and taurine (a key modulator of calcium accumulation) are important in promoting normal cell energetics. (4:55-60)" The claims cover only these three compounds. While Sole did mention the use of antioxidants, the only ones mentioned in his formula were Vitamin C and Vitamin E. There was no mention of lipoic acid, which works differently from Vitamins C and E and provides important additional actions which enhance the efficacy of the herein claimed invention. Sole claimed success with his cocktail and did not indicate any need to change it or substitute ingredients. Therefore, there is no motivation to combine with another reference.

The Office Action summarized Ames as teaching the combination of acetyl-L-carnitine and antioxidant such as lipoic acid to rejuvenate the mitochondria in aging animals. Ames does not suggest the use of other compounds, such as creatine or coenzyme Q. Therefore, Ames does not provide a suggestion or motivation to combine the two references.

In conclusion, Applicant respectfully requests that the Section 103 rejection be withdrawn.

Conclusion

The amendments here presented are made for the purposes of better defining the invention, rather than to overcome a rejection for patentability. No presumption should therefore attach that the claims have been narrowed over those earlier presented, or that subject matter or equivalents thereof to which the Applicant is entitled has been surrendered. No new matter has been introduced by these amendments.

Reconsideration and allowance is respectfully requested in view of the amendments and the following remarks.

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Claims:

1. (amended) A pet food for older pets [with anti-aging properties], the food comprising
 - a. an effective amount of lipoic acid [a suitable antioxidant];
 - b. an effective amount of a carnitine;
 - c. an effective amount of coenzyme Q;
 - d.[c.] carbohydrate;
 - e.[d.] protein;
 - f.[e.] fat; and
 - g.[e.] fiber.
2. (amended) The pet food of claim 1 wherein the carnitine is [ALC] acetyl-L-carnitine and the effective amount is about 0.1 grams to about 3 grams.
3. (amended) The pet food of claim 1 in which lipoic acid [antioxidant] is R- α -lipoic acid in the amount of about 0.1 grams to about 1.5 grams.
4. (amended) The pet food of claim 1, wherein the [further comprising] coenzyme Q is present in the amount of at least about 1 mg [/day].
5. (amended) The pet food of claim 1, further comprising creatine in the amount of at least about 0.2 grams [/day].
6. (amended) A pet treat for older pets [with anti-aging properties], the treat comprising
 - a. an effective amount of lipoic acid [a suitable antioxidant];
 - b. an effective amount of a carnitine;
 - c. an effective amount of coenzyme Q;
 - d.[c.] at least one energy source selected from carbohydrate, sugars, fat or a combination thereof; and
 - e.[d.] flavors.

7. (amended) The pet treat of claim 6 wherein the carnitine is [ALC] acetyl-L-carnitine and the effective amount is about 0.1 grams to about 3 grams.
8. (amended) The pet treat of claim 6 in which the lipoic acid [antioxidant] is R- α -lipoic acid in the amount of about 0.1 grams to about 1.5 grams.
9. (amended) The pet treat of claim 6, wherein the [further comprising] coenzyme Q is present in the amount of at least about 1 mg [/day].
10. (amended) The pet treat of claim 6, further comprising creatine in the amount of at least about 0.2 grams [/day].
11. (amended) A pet supplement for older pets [with anti-aging properties], the supplement comprising
 - a. an effective amount of lipoic acid [a suitable antioxidant]; [and]
 - b. an effective amount of coenzyme Q; and
 - c.[b.] an effective amount of a carnitine.
12. (amended) The pet food of claim 11 wherein the carnitine is [ALC] acetyl-L-carnitine and the effective amount is about 0.1 grams to about 3 grams.
13. (amended) The supplement of claim 11 in which the lipoic acid [antioxidant] is R- α -lipoic acid and is present in the amount of about 0.1 grams to about 1.5 grams.
14. (amended) The supplement of claim 11, in which the [further comprising] coenzyme Q is present in the amount of at least about 1 mg [/day].
15. (amended) The supplement of claim 11, further comprising creatine in the amount of at least about 0.2 grams [/day].

Feeding acetyl-L-carnitine and lipoic acid to old rats significantly improves metabolic function while decreasing oxidative stress

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Contributed by Bruce N. Ames, December 28, 2001

Mitochondrial-supported bioenergetics decline and oxidative stress increases during aging. To address whether the dietary addition of acetyl-L-carnitine [ALCAR, 1.5% (wt/vol) in the drinking water] and/or (R)- α -lipoic acid [LA, 0.5% (wt/wt) in the chow] improved these endpoints, young (2–4 mo) and old (24–28 mo) F344 rats were supplemented for up to 1 mo before death and hepatocyte isolation. ALCAR+LA partially reversed the age-related decline in average mitochondrial membrane potential and significantly increased ($P = 0.02$) hepatocellular O_2 consumption, indicating that mitochondrial-supported cellular metabolism was markedly improved by this feeding regimen. ALCAR+LA also increased ambulatory activity in both young and old rats; moreover, the improvement was significantly greater ($P = 0.03$) in old versus young animals and also greater when compared with old rats fed ALCAR or LA alone. To determine whether ALCAR+LA also affected indices of oxidative stress, ascorbic acid and markers of lipid peroxidation (malondialdehyde) were monitored. The hepatocellular ascorbate level markedly declined with age ($P = 0.003$) but was restored to the level seen in young rats when ALCAR+LA was given. The level of malondialdehyde, which was significantly higher ($P = 0.0001$) in old versus young rats, also declined after ALCAR+LA supplementation and was not significantly different from that of young unsupplemented rats. Feeding ALCAR in combination with LA increased metabolism and lowered oxidative stress more than either compound alone.

Harmon, Miguel, and others (1, 2) postulated that mitochondrial decay is a significant factor in aging, caused, in part, by the release of reactive oxygen species (ROS) as by-products of mitochondrial electron transport. Mitochondria are targets of their own oxidant by-products. The steady-state oxidative damage in mitochondria is high relative to other organelles, and the percentage of oxygen converted to superoxide increases with age (3–6). This leads to a vicious cycle of increasing mitochondrial damage, which adversely affects cell function (7), and results in a loss of ATP-generating capacity, especially in times of greater energy demand, thereby compromising vital ATP-dependent reactions. Cellular processes affected by mitochondrial decay include detoxification, repair systems, DNA replication, osmotic balance, and higher-order processes (7), such as cognitive function (7–9). Thus, preservation of mitochondrial function is important for maintaining overall health during aging (7). This theory is buttressed by the observation that caloric restriction, the only known regimen to increase mean life span in animals, maintains mitochondrial function and lowers oxidant production (7, 8, 10–12). A spartan diet of calorie restriction appears to be too unappealing to be widely adopted in humans, and thus other alternative regimens to improve or maintain normal mitochondrial activities have been sought.

Several dietary supplements, including the mitochondrial cofactor and antioxidant lipoic acid (LA), increase endogenous

antioxidants or mitochondrial bioenergetics (13–15). Feeding old rats acetyl-L-carnitine (ALCAR), a mitochondrial metabolite, reverses the age-related decline in tissue carnitine levels and improves mitochondrial fatty acid β -oxidation in the tissues studied (15–18). ALCAR supplementation also reverses the age-related alterations in fatty acid profiles and loss in cardiolipin levels, an essential phospholipid required for mitochondrial substrate transport (15–17). We demonstrated that ALCAR supplementation reverses the age-associated decline in metabolic activity in rats, suggesting that ALCAR improves mitochondrial function and increases general metabolic activity (19, 20). ALCAR-induced improvement in metabolic parameters appear to be responsible for improving short-term memory deficits and cognitive function in elderly subjects given ALCAR (21, 22) and in old rats (9).

This increased metabolic activity may come at a price, however, because supplementing rats with high levels of ALCAR lowered hepatocellular antioxidant status (19). This ALCAR-induced antioxidant loss was not seen, however, in other organs (T.M.H. and D. Heath, unpublished work) or when lower doses were given (23). We also showed that giving high [1.5% (wt/vol)], but not lower [0.5% (wt/vol)], supplemental doses of ALCAR to old rats increased mitochondrial oxidant flux, suggesting that while high ALCAR supplementation may increase electron flow through the electron transport chain, it also heightens formation of ROS as a consequence. We thus hypothesized that ALCAR supplemented with an antioxidant may have the salutary effect of increasing mitochondrial function and general metabolic activity without a concomitant increase in oxidative stress. We chose LA as a cosupplement for two reasons: (i) it is a naturally occurring cofactor for mitochondrial α -keto acid dehydrogenases (24), which may aid in cellular glucose-dependent ATP production (25); and (ii) in its reduced form, LA is a potent antioxidant and also increases intracellular ascorbate and glutathione concentrations (15, 26). Thus, LA and ALCAR may act together to reverse age-related metabolic decline and also reduce indices of oxidative stress.

We show that the combined supplementation of ALCAR and LA (ALCAR+LA) reverses age-related metabolic decline, improves hepatocellular ascorbate levels, and lowers oxidant appearance and oxidative damage.

Abbreviations: ALCAR, acetyl-L-carnitine; LA, lipoic acid; MDA, malondialdehyde; ROS, reactive oxygen species.

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Materials and Methods

The following chemicals were used: EGTA, trypan blue, glutathione, heparin (sodium salt), and rhodamine 123 (R123) (Sigma); 2',7'-dichlorofluorescein diacetate (Molecular Probes); collagenase (type D) (Roche Molecular Biochemicals); and LA and *meta*-phosphoric acid (Fluka). ALCAR was a gift of Sigma Tau (Pomezia, Italy) and purchased from Aldrich. LA was a gift from Hans Tritschler, Asta Medica, Frankfurt. All other reagents were reagent grade or better. Double-distilled/deionized water was used throughout.

Rats (Fischer 344, virgin male, outbred albino) (3–5 mo) were obtained from Simonsen Laboratories (Gilroy, CA). Old rats (identical strain; 20–28 mo) were from the National Institute on Aging animal colonies. All animals were fed Purina rodent chow and water ad libitum. There was no discernable difference in food consumption in ALCAR vs. untreated rats. All animals were acclimatized at the Northwest Animal Facilities at the University of California at Berkeley for at least 1 week before experimentation.

ALCAR Supplementation. Old and young rats were given a 1.5% (wt/vol; pH adjusted to ≈ 6) solution of ALCAR in their drinking water and allowed to drink ad libitum for 1 mo before death and hepatocyte isolation. Both young and old rats typically drank ≈ 20 ml/rat per day (data not shown), which provided a daily ALCAR dose of ≈ 0.75 g/kg body wt per day for old rats and 1.2 g/kg body wt per day for young rats. All animal experiments were done with appropriate Animal Use Committee clearances.

LA Supplementation. Young and old rats were given LA [0.5% (wt/wt)] mixed into the AIN-93M chow (Dyets, Bethlehem, PA) for 2 weeks before death. Unsupplemented animals were fed Purina rodent chow and water ad libitum. The pellets were made into a mush and fed to some young and old rats for 2 weeks before cell isolation. Both young and old rats typically ate ≈ 15 g/rat per day (data not shown), which provides a daily LA dose of 0.12 g/kg body weight for young rats and 0.075 g/kg body weight for old rats.

Cell Isolation. Liver tissue was dispersed into single cells by collagenase perfusion (27). Cell number was assessed by using a hemocytometer, and viability was determined by trypan blue exclusion. Viability was usually more than 90% in both age groups.

Flow Cytometry. Hepatocytes (2.0×10^6 cells) were incubated with R123 (0.01 mg/ml) for 30 min at 37°C and then subjected to flow cytometry (described in ref. 20).

Assay of Oxidants with 2',7'-Dichlorofluorescein Diacetate. Formation of oxidants in cells was determined by fluorescence over time by using 2',7'-dichlorofluorescein diacetate, a reduced, nonfluorescent derivative of fluorescein (28). Quadruplicate samples were routinely analyzed. Fluorescence was monitored by using a Cytofluor 2350 fluorescent measurement system (Millipore) with standard fluorescein filters and CYTOCALC software. Because the majority of cells from old rats consume oxygen at lower rates than cells from young animals (20), the rate of oxidant production was normalized to the level of oxygen consumed. Cellular oxygen consumption was measured by using a Yellow Springs Instruments 5300 oxygen electrode and monitor. Cells (4.0×10^6) were added to 3 ml of Krebs–Henseleit balanced salt medium supplemented with 1 mM glucose and 7 mM glutamate, pH 7.4, that had been previously equilibrated to 20°C, and oxygen consumption was monitored for at least 15 min.

Ascorbate Analysis. Ascorbic acid analysis was performed as described (29). Briefly, samples were acidified with *meta*-phosphoric acid (10% wt/vol) and mixed with 50 μ l of 200 mM Trizma buffer (Sigma), pH 9.0, giving a sample pH of about 2.5. The samples were placed in a chilled (2°C) auto sampler for analysis. The system used for separation was reversed-phase HPLC (Hewlett–Packard) with coulometric detection (ESA, Bedford, MA). The peak area corresponding to ascorbic acid was integrated by using Hewlett–Packard CHEMSTATION software and compared with a standard curve based on authentic material.

Malondialdehyde (MDA) Measurement. Lipid peroxidation was assayed by using a gas chromatography–MS method for MDA (30, 31). Briefly, hepatocytes were lysed with PBS containing 2.8 mM butylated hydroxytoluene and 1% (wt/vol) SDS, pH 7.4. The protein-bound MDA was hydrolyzed with H_2SO_4 . MDA was converted to a stable derivative by using pentafluorophenyl hydrazine (room temperature). The derivative was detected with a Hewlett–Packard 5890 Series II gas chromatograph interfaced to a 5989 MS system equipped with a J & W Scientific (Folsom, CA) DBWAX capillary column (15 m \times 0.25 mm i.d., 0.25- μ m film thickness) in the negative chemical ionization mode. The results were indexed to protein, which was measured by using the BCA protein assay kit (Pierce).

Activity Tests. Ambulatory activity was monitored as described (18). Briefly, each night rats were moved from group housing to individual cages (48 cm l \times 25 cm w \times 20 cm h) at least 4 h before the quantification of ambulatory parameters. The room was on a 12 h light/dark cycle (lights on 6 a.m. to 6 p.m.). At 8 p.m. dim light illuminated the test subjects for video tracking. Quantification began at 9 p.m. and continued for 4 h. One hour later the low light turned off, and the room remained in total darkness until 6 a.m. when the standard light/dark cycle lighting began. A video signal from a camera suspended directly above the individual cages was fed directly into a Videomex-V (Columbus Instruments, Columbus, OH) computer running the MULTIPLE OBJECTS MULTIPLE ZONES software. The system quantified ambulatory activity parameters and was calibrated to report distance traveled in cm. In addition to total distance traveled, the time each subject spent in ambulatory (locomotor), stereotypic (grooming), and resting (nonmovement) activity was recorded and dumped in hourly segments to an IBM computer. This hourly data were recorded by the Videomex-V MULTIPLE OBJECTS MULTIPLE ZONES monitoring software. At 9 a.m. animals were removed from individual housing and returned to group housing. Before ALCAR+LA supplementation and for 2 consecutive nights, the ambulatory activity of each rat was recorded. After ALCAR+LA supplementation and for 2 consecutive nights, the same spontaneous locomotor parameters were determined. Each rat served as its own control.

Statistical Analysis. Statistical significance was determined by using the paired two-tailed Student's *t* test or single factor ANOVA. Results are expressed as the mean \pm SE.

Results

Measurement of Cellular and Physiological Parameters of Metabolic Activity. To determine whether cosupplementation of ALCAR+LA improved general parameters of physiological activity, a series of experiments were performed to monitor indices of cellular and whole animal metabolism.

We previously showed significantly lower average mitochondrial membrane potential ($\Delta\Psi$) in the majority of hepatocytes from old rats compared with young rats, but a 1-mo feeding regimen of 1.5% (wt/vol) ALCAR reversed this decline in $\Delta\Psi$ (19). For the present study, we also found a marked age-related

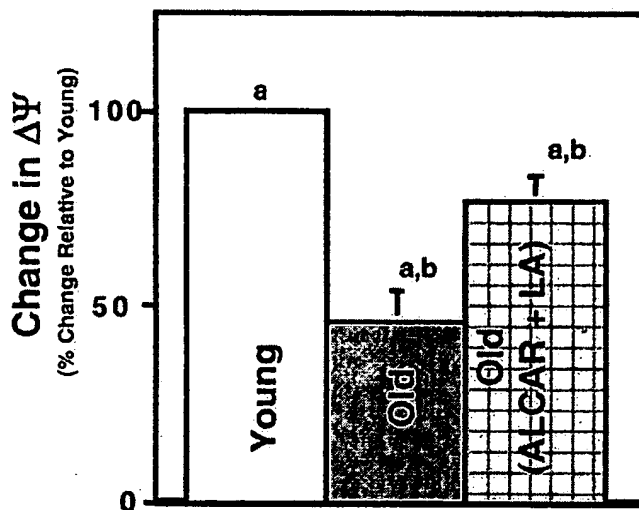


Fig. 1. Dietary supplementation of ALCAR+LA partially reverses the age-related decline in average mitochondrial $\Delta\Psi$. Rats were fed ALCAR+LA as described, and average hepatocellular $\Delta\Psi$ was assessed by using R123. Results showed that average $\Delta\Psi$ was significantly lower in cells from old animals relative to young. However, ALCAR+LA supplementation significantly increased average $\Delta\Psi$ over that seen in unsupplemented old rats. Columns denoted by the same letter are statistically different ($P \leq 0.05$) from each other.

decline in this key parameter of mitochondrial function (Fig. 1). Relative to mean fluorescence characteristics seen in hepatocytes from young unsupplemented animals, the average $\Delta\Psi$ for hepatocytes from old rats was $53.8 \pm 8.0\%$ lower ($n = 5$), representing a significant loss ($P = 0.02$). Feeding ALCAR+LA to old rats markedly reversed this decline (Fig. 1). Old rats on the ALCAR+LA supplemented diet had an average $\Delta\Psi$ that was only $22.8 \pm 6.0\%$ lower relative to young unsupplemented rats. Thus, dietary supplementation with ALCAR+LA partially restored the loss of mitochondrial $\Delta\Psi$ although the improvement was not as great as previously observed with ALCAR alone (19).

We previously showed in separate reports that ALCAR or LA supplementation increased hepatocellular and myocardial oxygen consumption, indicating that either compound was able to increase cellular metabolism (18, 19). Young and old rats were supplemented with or without ALCAR+LA before cell isolation, and this general parameter of metabolic rate was monitored by using an oxygen electrode. Hepatocellular oxygen consumption declined from 1.03 ± 0.17 ($n = 5$) to 0.54 ± 0.09 $\mu\text{mol}/\text{min}$ per 10^6 cells ($n = 5$) in young versus old unsupplemented rats, a significant ($P = 0.03$) decline of 47.6% with age. These results are in agreement with our previous results and suggest that there is an age-related decline in hepatocellular metabolic rate. Oxygen consumption in hepatocytes from old rats treated with ALCAR+LA was 0.82 ± 0.07 $\mu\text{mol O}_2/\text{min}$ per 10^6 cells versus 0.95 ± 0.05 $\mu\text{mol}/\text{min}$ per 10^6 in unsupplemented ($n = 5$) young rats ($P = 0.02$). Thus, feeding ALCAR+LA to old rats significantly reversed the age-related decline in hepatocellular oxygen consumption.

Ambulatory Activity. To further explore whether ALCAR+LA generally improved metabolic rate on a whole animal basis, we studied ambulatory activity in animals fed with or without ALCAR+LA. Old rats exhibited a 3-fold decline in ambulatory activity in terms of overall movement and the amount of time spent in movement (Table 1). The speed of old animals when in movement was not different from that shown by young animals, suggesting that the age-related decline in activity was not caused

Table 1. Ambulatory activity in rats fed with or without ALCAR+LA

	Young	Old	Percent difference
Ambulatory activity before ALCAR+LA supplementation			
Distance (cm/h)	528 ± 43	177 ± 19	-66.5
Ambulation (s)	58 ± 5	20 ± 2	-65.0
Resting (s)	$1,463 \pm 94$	$1,717 \pm 73$	17.4
Speed (cm/s)	9.1 ± 0.2	8.6 ± 0.2	—
Ambulatory activity after ALCAR+LA supplementation			
Distance (cm/h)	693 ± 60	376 ± 23	-45.7
Ambulation (s)	76 ± 7	43 ± 3	-43.4
Resting (s)	$1,397 \pm 55$	$1,982 \pm 60$	41.8
Speed (cm/s)	9.2 ± 0.1	8.8 ± 0.1	—
Percent change (pre- versus post-ALCAR+LA supplementation)			
Distance	31.3	112.4	
Ambulation	31.0	115.0	
Resting	-4.5	15.4	
Speed	1.1	2.3	

by pain or the inability to move, but rather it reflected a general loss of metabolic activity.

Animals were then fed ALCAR+LA for 1 mo (in the case of LA 2 weeks) and again tested for ambulatory activity. Results show that ALCAR+LA significantly improved ambulatory activity in young and old animals. For the young animals, the amount of active time and the overall distance traveled increased by $\approx 31\%$ when compared with their activity before ALCAR+LA supplementation. A much greater increase was observed in old rats. Ambulation and overall distance traveled more than doubled from 20 ± 2 s per movement and 177 ± 19 cm/h to 43 ± 3 s per movement and 376 ± 23 cm/h, respectively. This increase, although still not as good, on average, as young untreated rats, nevertheless represented a significant ($P = 0.03$) improvement versus that of old untreated animals. Thus, ALCAR+LA supplementation not only reverses the age-related decline in oxygen consumption, a cellular parameter of metabolic activity, but also increases ambulatory activity, a general physiological parameter of metabolic activity.

Antioxidant Status/Oxidative Stress. We previously observed that feeding 1.5% (wt/vol) ALCAR alone to old rats, although markedly increasing metabolic activity through improved mitochondrial function, also resulted in heightened oxidant production and decreased low molecular weight antioxidant status. This finding was presumably caused by increased formation of ROS/reactive nitrogen species as by-products of heightened metabolic activity. To understand whether feeding ALCAR+LA could ameliorate this potential increase in oxidative stress, we measured ascorbic acid status, overall oxidant production, and markers of oxidative damage in freshly isolated hepatocytes taken from young and old rats fed with or without ALCAR+LA.

Hepatocytes from old rats had significantly lower ascorbate levels as compared with young rats (7.29 ± 2.97 versus 3.38 ± 0.67 ; $P = 0.003$) (Fig. 2), suggesting that liver antioxidant status may be compromised with age. We observed, as previously, that ALCAR supplementation at 1.5% (wt/vol) resulted in a further and significant decline in ascorbate levels beyond the observed age-related loss in this key antioxidant. However, ALCAR+LA supplementation reversed the ALCAR-induced and age-related loss of ascorbate such that there was no longer a significant difference ($P = 0.3$) in hepatocellular ascorbate values between ALCAR+LA-treated old rats and that of untreated young animals (Fig. 2).

To further investigate whether ALCAR+LA actually affected oxidative stress parameters in old rats, hepatocellular oxidant

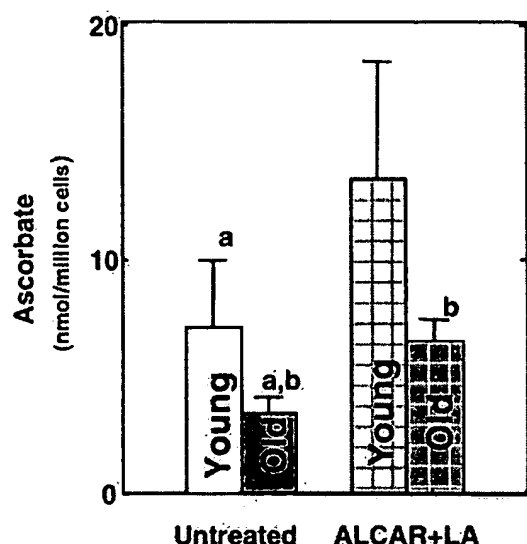


Fig. 2. LA supplementation reverses the ALCAR-induced and age-related decline in hepatocellular ascorbate levels. Hepatocytes (1.0×10^6 cells) were hydrolyzed in 10% (wt/vol) metaphosphoric acid, and the ascorbate content in the supernatant was analyzed by HPLC with electrochemical detection (31). Results show that old rats had significantly lower hepatocellular ascorbate concentrations than cells isolated from young animals. For rats fed 1.5% (wt/vol) ALCAR, ascorbate levels in hepatocytes from both young and old rats were significantly lower than corresponding controls (not shown). Cosupplementation of LA with ALCAR negated both the age-related and ALCAR-induced decline in hepatocellular ascorbate concentrations. Columns denoted by the same letter are statistically different ($P \leq 0.05$) from each other.

production was monitored by using 2',7'-dichlorofluorescein oxidation. This cell permeant dye becomes fluorescent when it is oxidized. Thus, general oxidant production can be monitored in cells by measuring the rate of increased fluorescence over time. Cells isolated from young and old rats exhibited a marked difference in fluorescence appearance (Fig. 3). Oxidant production increased over 30.8% with age from $2,942.3 \pm 99.3$ to $3,835.22 \pm 303.6$ fluorescence units/min per $\mu\text{mol O}_2$ consumed per 10^6 cells. This finding is in agreement with our previously published results (18) and is consistent with lower antioxidant status and heightened mitochondrial oxidant production during aging. Addition of ALCAR+LA to the diet of old rats caused a significant decline in appearance of oxidants to $2,801.79 \pm 308.0$ fluorescence units/min per $\mu\text{mol O}_2$ consumed per 10^6 cells, which was not different from untreated or ALCAR+LA-fed young rats. Thus, the combination of ALCAR with LA not only reverses the age-related increase in oxidants, but also the additional oxidants induced by high doses of ALCAR. These results suggest that ALCAR+LA supplementation not only improves metabolic rate and physiological activity, but does so without causing a concomitant increase in oxidants.

To further assess whether ALCAR+LA modulated age-related and ALCAR-induced oxidative stress, we also measured steady-state levels of MDA, a marker of lipid peroxidation (Fig. 4). Hepatocellular MDA levels in old untreated rats were more than 4-fold higher than the levels seen in young rats, a significant increase ($P = 0.0001$). Similar to results shown for oxidant production, we observed a small, but significant, increase in steady-state MDA levels in liver tissue from old rats fed ALCAR alone (Fig. 4); on average, a similar increase in young rats was not significant. These results again suggest that high ALCAR alone, although improving metabolism and cognitive function, also increased oxidative stress in the liver. When LA was given along with ALCAR, we observed that there was a significant decline in MDA levels (Fig. 4). Most importantly, hepatic MDA

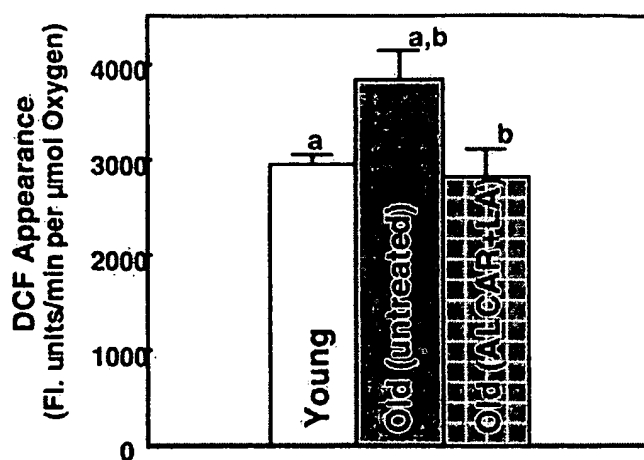


Fig. 3. ALCAR+LA supplementation lowers 2',7'-dichlorofluorescein (DCF) appearance. Young and old rats were treated with or without ALCAR+LA, and hepatocytes were isolated as described. Cells ($40,000$) were incubated with dichlorofluorescein diacetate ($20 \mu\text{M}$, final concentration), and the rate of fluorescence appearance was measured by using a Cytofluor fluorescent plate reader (as described in *Materials and Methods*). Because of the difference in oxygen consumption characteristics, all rates of fluorescence were expressed in relation to oxygen consumption. Results show that ALCAR+LA supplementation significantly lowered the age-related increase in DCF appearance, indicating lower ROS and/or reactive nitrogen species in hepatocytes under this feeding regimen. Columns denoted by the same letter are statistically different ($P \leq 0.05$) from each other.

concentrations in old ALCAR+LA fed rats no longer statistically differed from those found in young untreated animals.

Discussion

We previously demonstrated that feeding old rats ALCAR markedly improves the average mitochondrial membrane potential, a key indicator of mitochondrial function, to a level no longer significantly different from that of young rats (18). This reversal of membrane potential appears to be caused, in part, by replenishment of carnitine, a betaine that shuttles fatty acids into the mitochondrion for β -oxidation. ALCAR administration also appears to reverse the age-related decline in cardiolipin levels. Cardiolipin is a key phospholipid cofactor for a number of mitochondrial substrate transporters as well as the protein complexes in the electron transport chain. Thus, age-related decline in cardiolipin could profoundly and adversely affect mitochondria.

Our results, in combination with studies by Hagen, Paradies, Gadaleta, and others (15, 16), clearly demonstrate that ALCAR improves metabolic function in a number of tissues, most likely by improving substrate and electron flux through mitochondria.

ALCAR does not, however, improve one aspect of mitochondrial decay in old rats, namely, the age-related increase in oxidants. Electron transfer through the mitochondrial electron transport chain becomes less efficient with age, which leads to increased oxidant leakage. ALCAR at the 1.5% level used in our initial experiments appears to increase electron flow through the electron transport chain, which further increases the appearance of ROS. In support of this concept, we observed higher oxidant appearance and lower hepatocellular antioxidant status after ALCAR supplementation (18). Feeding 1.5% ALCAR to old rats improved the age-related decline in metabolic rate, but increased oxidant appearance to a small, yet significant, degree. This ALCAR-induced increase in hepatocellular oxidative stress may be unique to the liver or caused by the relatively high levels of ALCAR used in this feeding study. In recent studies where old rats were fed 1.5% ALCAR, we did not observe any ALCAR-

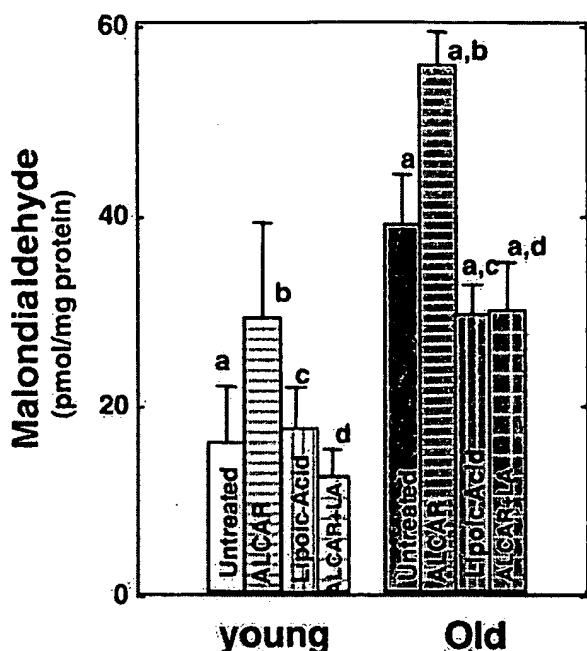


Fig. 4. ALCAR+LA supplementation significantly lowers the steady-state age-related accumulation of lipid peroxidation. MDA, a marker of lipid peroxidation, was measured by gas chromatography/MS as described (29, 30). Results show no statistical increase ($P \geq 0.05$) in MDA levels with ALCAR, LA, or both supplements in liver tissue from young animals. MDA levels were significantly increased in liver from old rats relative to young controls, and ALCAR supplementation alone further increased hepatic MDA content over that of nonsupplemented old rats. LA alone and ALCAR+LA supplementation significantly decreased hepatic MDA content in livers from old rats. Columns denoted by the same letter are statistically different ($P \leq 0.05$) from each other.

induced increased oxidative stress in the heart but saw a significant improvement in mitochondrial function and cellular metabolism (T.M.H., J. Suh, and D. Heath, unpublished results). In other studies using lower ALCAR doses [0.5% (wt/vol)], Liu *et al.* (22) noted no ALCAR-induced changes in parameters of oxidative stress in rat brain, yet found that this dose significantly improved cognitive function in aged animals (9). Thus, smaller doses of ALCAR may effectively improve metabolic function without higher oxidative stress.

The rationale for the present study was to determine whether other mitochondrial metabolites fed along with ALCAR could improve metabolic parameters and lower the age-related increase in oxidative stress. We chose to cosupplement LA with ALCAR because LA is easily taken up into a variety of tissues and can be reduced to a powerful antioxidant, dihydrolipoic acid (23). Even though LA/dihydrolipoic acid is quickly removed from most cells, this compound also induces cystine/cysteine uptake and can thereby increase glutathione synthesis (25). LA supplementation maintains and actually reverses the age-related

decline in hepatocellular and myocardial ascorbate and glutathione levels, even when cells were incubated with *tert*-butylhydroperoxide, a model alkyl peroxide (32, 33). Thus, LA may not only act synergistically with ALCAR to improve mitochondrial-supported bioenergetics but may also improve general antioxidant status, which declines with age.

LA elicits other cell responses that may complement the actions of ALCAR on the cell. LA enhances glucose uptake by increasing glucose transporters at the surface of cells (24). It is also a cofactor for α -keto acid dehydrogenases found in the mitochondria, and its supplementation in the diet of aging animals may thus correct any age-associated decline in α -ketoglutarate dehydrogenase and pyruvate dehydrogenase caused by lost cofactors. Humphries and Szveda (34) showed that pyruvate dehydrogenase and α -ketoglutarate dehydrogenase can be modified by adduction with 4-hydroxy-nonenal (R. Moreau and T. M. Hagen, personal communication), rendering it unable to transfer acetyl groups. MDA and 4-hydroxy-nonenal also inactivate carnitine acyltransferase and decrease the binding affinity for substrates (35). Thus, LA may act synergistically with ALCAR to improve both fatty acid and glucose catabolism and energy production. Indeed, we have previously shown that LA alone also increases oxygen consumption and mitochondrial membrane potential, although not as effectively as ALCAR (14).

Supplementing the diet of old rats with ALCAR+LA significantly improves many of the most frequently encountered age-related changes in mammals—namely loss of energy metabolism, increased oxidative stress, decreased physical activity, and as shown in ref. 9, impaired cognitive function. This affect on cognitive function has been previously observed for both ALCAR and LA (36), but to our knowledge, has not been observed for the combination of the two supplements. How ALCAR and LA affect short-term memory is not well understood, but may be caused by a number of factors, including increased neurotransmitter production, improved mitochondrial function, and/or calcium handling by the neuron (20, 21, 36). We have also recently found that LA alone significantly reduces the age-related accumulation of iron and copper in the brain (J. H. Suh, personal communication). Thus, the LA component may also increase neuro-cognitive function by potentially lowering iron and copper-induced oxidative stress.

Presently, only short-term feeding regimens of ALCAR+LA have been given to aged animals. The present study suggests that long-term feeding experiments are warranted to monitor how effectively ALCAR+LA supplementation ameliorates oxidative stress, loss of metabolic function, and mild cognitive impairment seen in older animals. In this regard, it is of interest that many of the cellular effects of caloric restriction, which does increase lifespan, are also affected by ALCAR+LA supplementation.

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Age-associated mitochondrial oxidative decay: Improvement of carnitine acetyltransferase substrate-binding affinity and activity in brain by feeding old rats acetyl-L-carnitine and/or *R*- α -lipoic acid

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We test whether the dysfunction with age of carnitine acetyltransferase (CAT), a key mitochondrial enzyme for fuel utilization, is due to decreased binding affinity for substrate and whether this substrate, fed to old rats, restores CAT activity. The kinetics of CAT were analyzed by using the brains of young and old rats and of old rats supplemented for 7 weeks with the CAT substrate acetyl-L-carnitine (ALCAR) and/or the mitochondrial antioxidant precursor *R*- α -lipoic acid (LA). Old rats, compared with young rats, showed a decrease in CAT activity and in CAT-binding affinity for both substrates, ALCAR and CoA. Feeding ALCAR or ALCAR plus LA to old rats significantly restored CAT-binding affinity for ALCAR and CoA, and CAT activity. To explore the underlying mechanism, lipid peroxidation and total iron and copper levels were assayed; all increased in old rats. Feeding old rats LA or LA plus ALCAR inhibited lipid peroxidation but did not decrease iron and copper levels. *Ex vivo* oxidation of young-rat brain with Fe(II) caused loss of CAT activity and binding affinity. *In vitro* oxidation of purified CAT with Fe(II) inactivated the enzyme but did not alter binding affinity. However, *in vitro* treatment of CAT with the lipid peroxidation products malondialdehyde or 4-hydroxy-nonenal caused a decrease in CAT-binding affinity and activity, thus mimicking age-related change. Preincubation of CAT with ALCAR or CoA prevented malondialdehyde-induced dysfunction. Thus, feeding old rats high levels of key mitochondrial metabolites can ameliorate oxidative damage, enzyme activity, substrate-binding affinity, and mitochondrial dysfunction.

Aging appears to be due, in part, to damage caused by the oxidants produced by mitochondria as by-products of normal metabolism (1–10). Aging is associated with a decrease in cellular enzyme or receptor activities. Some enzyme or receptor inactivation is due to an increase in K_m for their substrates or cofactors (B.N.A., J.L., and I. Elson-Schwab, unpublished work). For example, Feuers (11) found that in female mice, the V_{max} of mitochondrial complexes III and IV significantly decreased with age, in parallel with a decrease of ubiquinol or cytochrome *c* substrate-binding affinity. Dietary restriction, which reduces the generation of oxidants and oxidative damage, effectively reversed these decreases in complex activity and substrate affinity. On the other hand, the activity of many enzymes decreases with age but shows no change in K_m (12–14) (B.N.A., J.L., and I. Elson-Schwab, unpublished work).

As many as one-third of mutations in a gene result in the corresponding enzyme having a poorer binding affinity (an increased K_m) for its coenzyme, which in turn lowers the rate of the reaction (15–17). When the concentration of the coenzyme is increased by feeding the corresponding vitamin at high levels, the enzyme activity is partially restored, and the disease phenotype is cured or ameliorated (18). Thus, we hypothesize (18–20) that

during aging, mitochondrial oxidants deform proteins because of direct oxidation, changes in membranes, and adduction of aldehyde by-products from lipid peroxidation. This deformation in turn decreases the binding affinity of many enzymes for their substrates or coenzymes. Feeding high doses of enzyme substrates or coenzymes can overcome the deficiencies of those enzymes with decreased binding affinity and restore enzyme function. Oxidative decay is particularly acute in mitochondria (1, 4, 8). Thus, feeding high levels of several mitochondrial substrates and vitamin precursors of coenzymes might reverse some of the mitochondrial decay of aging (5–7, 9, 18, 21, 22).

L-Carnitine is a betaine required in the mitochondria for transporting in long chain fatty acids for β oxidation and ATP production, as well as for transporting out excess short and medium chain fatty acids (23). Feeding old rats an acetyl-L-carnitine (ALCAR)-supplemented diet restores tissue levels of free and acyl carnitines to that found in plasma and brain tissues of younger animals (20, 24). This diet-induced increase in carnitine levels in older animals results in a reversion of liver and heart mitochondria to a more youthful state, both structurally and functionally (6, 9, 14, 25–28).

R- α -lipoic acid (LA) is a coenzyme for pyruvate dehydrogenase and α -ketoglutarate dehydrogenase in mitochondria. Dihydrolipoic acid, the reduced form of LA, is a potent antioxidant that can recycle other antioxidants, such as vitamins C and E, and raise the levels of intracellular glutathione, which is critical for neuronal function (29, 30). LA supplementation restores long-term potentiation, a synaptic analogue of learning and memory, in aged rodents (31) and partially restores ambulatory activity and memory lost during aging (5, 32, 33).

Carnitine acetyltransferase (CAT) (EC 2.3.1.7) catalyses the reversible conversion of acetyl-CoA and carnitine to acetylcarnitine and CoA. CAT's essential functions are to regenerate CoA, which allows peroxisomal β -oxidation to proceed, and to facilitate transport of acetyl moieties to mitochondria for oxidation (34). More than 70% of CAT is located in the mitochondrial matrix, and it appears to be present in all mammalian tissues (34–36). An age-associated decrease of CAT activity has been reported in rat soleus, diaphragm, and heart (37, 38) and in brain and muscles in vitamin E-deficient rats (39), although Moret *et*

Abbreviations: ALCAR, acetyl-L-carnitine; CAT, carnitine acetyltransferase; HNE, 4-hydroxynonenal; LA, *R*- α -lipoic acid; MDA, malondialdehyde; TBS, Tris-buffered saline.

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al. (40) did not find altered CAT activity in the brain of Long-Evans rats with age in healthy animals over a moderate age range. CAT activity was found to decrease in Alzheimer's patient brain microvessels and cerebellum (41, 42), although there are contrary findings (43). CAT activity was also found to decrease in fatal ataxic encephalopathy (44), mitochondrial encephalomyopathy (45), and severe peripheral vascular disease (46). Sulfhydryl reactive agents cause a decrease in CAT-binding affinity for substrates (increase in K_m) and in CAT activity, and the addition of CAT substrates or antioxidants such as mercaptoethanol prevents or partially reverses CAT inhibition (47). No study has attempted to examine the age-associated changes in CAT substrate-binding affinity and the effects of substrates or antioxidant treatments on the K_m of CAT.

The present study was designed to test the K_m hypothesis by assaying the kinetics of CAT in the brains of young and old rats, and old rats fed ALCAR and/or LA. The mechanism for the change in kinetics was also explored.

Materials and Methods

Materials. ALCAR (hydrochloride salt) was a gift of Sigma Tau (Pomezia, Italy) and LA (the natural *R*-isomer), of ASTA Medica (Frankfurt/Main, Germany). All other reagents were reagent grade or the highest quality available from Sigma, unless otherwise indicated.

Animals. Fischer 344 male rats were obtained from the National Institute on Aging. Control animals were fed an AIN93M diet from Dyets (Bethlehem, PA) and MilliQ (Millipore) water (pH 5.2). The rats in the experimental groups were fed either 0.5% ALCAR in their drinking water, 0.2% LA in AIN93M diet, or both, for 7 weeks. The young rats were 4.5 months, and the old ones were 24.5 months at the start of experiment; they were more than 7 weeks older when they were killed with ether anesthesia. The brains were removed, immediately put into liquid nitrogen, and stored in a -80°C freezer until analysis.

Kinetic Analysis. Because there are no brain regional differences in CAT activity as well as K_m in rats (40) or in rabbits (36), we used the whole brain. Brain tissue was homogenized with 50 mM Tris-buffered saline (pH 7.5) containing 2 mM EDTA, 5 mM MgCl_2 , 0.8 mM DTT, 1 μM protease inhibitor mixture, and 0.25 mM phenylmethylsulfonyl fluoride (freshly made in acetone and added to the homogenizing tube before homogenization). Homogenates were then sonicated on ice for 3×10 s and centrifuged at $3,500 \times g$ for 5 min to obtain the mitochondrial and microsomal portion containing more than 90% of the enzyme (36). The CAT activity was assayed immediately after the centrifugation as described (48, 49). The assay medium contained about 0.5 mg of protein/ml brain homogenate supernatant, 50 mM Tris, 2 mM EDTA, 25 mM malate, 0.25 mM NAD, 12.5 $\mu\text{g}/\text{ml}$ of rotenone, 12.5 $\mu\text{g}/\text{ml}$ of malate dehydrogenase, 50 $\mu\text{g}/\text{ml}$ of citrate synthase, and 0.04% Triton-100. The kinetics were determined over a range of ALCAR concentrations from 0.015 to 5 mM with a constant concentration of 1.25 mg/ml of CoA (K_m for ALCAR) or over a range of CoA concentrations from 6.25 to 400 μM with a constant concentration of 2 mM acetyl-L-carnitine (K_m for CoA). The results were plotted with the double-reciprocal plot of reciprocal rate $1/v$ against reciprocal substrate concentration $1/S$. Results were also calculated by the direct linear plot with the equation of $V_{\max} = v + (v/S)K_m$ (50).

Ex Vivo Oxidation of Rat-Brain Homogenate. The young-rat-brain homogenate was incubated with FeSO_4 for 15 min at 37°C . The kinetics were assayed as described above, and the oxidation was assayed by measuring malondialdehyde (MDA) with a gas chromatography-mass spectrometric method (51, 52).

In Vitro Oxidation of Purified CAT with Iron. CAT (purified from pigeon breast muscle) was obtained from Sigma. The enzyme was diluted with PBS and used immediately. The enzyme (0.36 $\mu\text{g}/\text{ml}$) was incubated with PBS and various concentrations of FeSO_4 with or without metal chelators/antioxidants at room temperature for 15 min. CAT activity was assayed immediately by using the method described above (CAT at 0.036 $\mu\text{g}/\text{ml}$ of protein).

MDA- and 4-Hydroxynonenal (HNE)-Induced Inactivation and K_m Change of Purified CAT in Vitro. MDA was prepared by derivatization of 1,1,3,3-tetramethoxypropane with 0.01 M HCl and stored 3–6 weeks at 4°C . The concentration of MDA was determined at 245 nm by using an extinction coefficient of 13,700/M (52). HNE was obtained from Calbiochem, and its concentration was determined at 224 nm by using an extinction coefficient of 13,750/M (53). Similar to the above incubation, CAT (0.36 $\mu\text{g}/\text{ml}$) was incubated in PBS or Tris-buffered saline (TBS) with MDA or HNE at room temperature. The CAT kinetics were assayed immediately as above (CAT at 0.036 $\mu\text{g}/\text{ml}$ of protein).

Total Metal Assay with Inductively Coupled Plasma Spectrometry (ICP). Six metals (iron, copper, calcium, magnesium, manganese, and zinc) were analyzed by using ICP with modification of a reported method (54, 55).

Lipid Peroxidation Assay. Lipid peroxidation was assayed by using a gas chromatography-mass spectrometric method to measure the level of MDA (51, 52).

Results

CAT Kinetics. The double-reciprocal plots of CAT reaction velocity versus ALCAR or CoA concentration in rat brain are shown in Fig. 1 A and B. The values of V_{\max} and apparent K_m are shown in Fig. 1 C and D. Compared with young rats, old rats showed a moderate decrease in enzyme activity (V_{\max}) (14%, Fig. 1C) and an increase in K_m [160% of K_m for ALCAR and 180% of K_m for CoA (Fig. 1D)], suggesting a decrease in substrate-binding affinity. Supplementation with ALCAR in old rats significantly increased the binding affinity for ALCAR. Supplementation with LA showed a small increase in binding affinity that was not statistically significant. The combination of ALCAR and LA significantly elevated enzyme activity and binding affinity for both substrates (K_m for ALCAR, $P = 0.019$; K_m for CoA, $P = 0.018$). The combination also significantly increased CAT activity ($P = 0.04$).

Ex Vivo Oxidation of Brain Homogenate from Young Rats. Incubation of Fe(II) (1–10 mM) with brain homogenate from young rats for 15 min induced a concentration-dependent inactivation of CAT. The inactivation was accompanied by a significant decrease in substrate-binding affinity (Fig. 2A), similar to that associated with aging (Fig. 1). Incubation of rat-brain homogenate with Fe(II) induced a marked increase in membrane lipid peroxidation, as shown by the level of MDA (control, 15.2 ± 0.2 ; addition of 0.2 mM FeSO_4 , 134 ± 1.6 pmol/mg of protein). As expected, metal chelators such as EDTA at 1 mM (MDA 48.6 ± 0.6 pmol/mg) and deferoxamine at 1 mM (MDA 8.5 ± 0.2 pmol/mg) protected lipid membranes from peroxidation.

In Vitro Oxidation of Purified CAT. Incubation of purified enzyme with Fe(II) (0.1–1 mM) induced a concentration-dependent inactivation of CAT with a 50% inactivation at a concentration of 95 μM at 37°C for 15 min (0.5 units/ml). Unlike *ex vivo* oxidation of rat-brain homogenate, this oxidation induced a decrease in enzyme activity but not in the substrate-binding affinity (Fig. 2B). FeSO_4 at 200 μM concentration caused 80% inactivation of CAT. As expected, metal chelators EDTA and

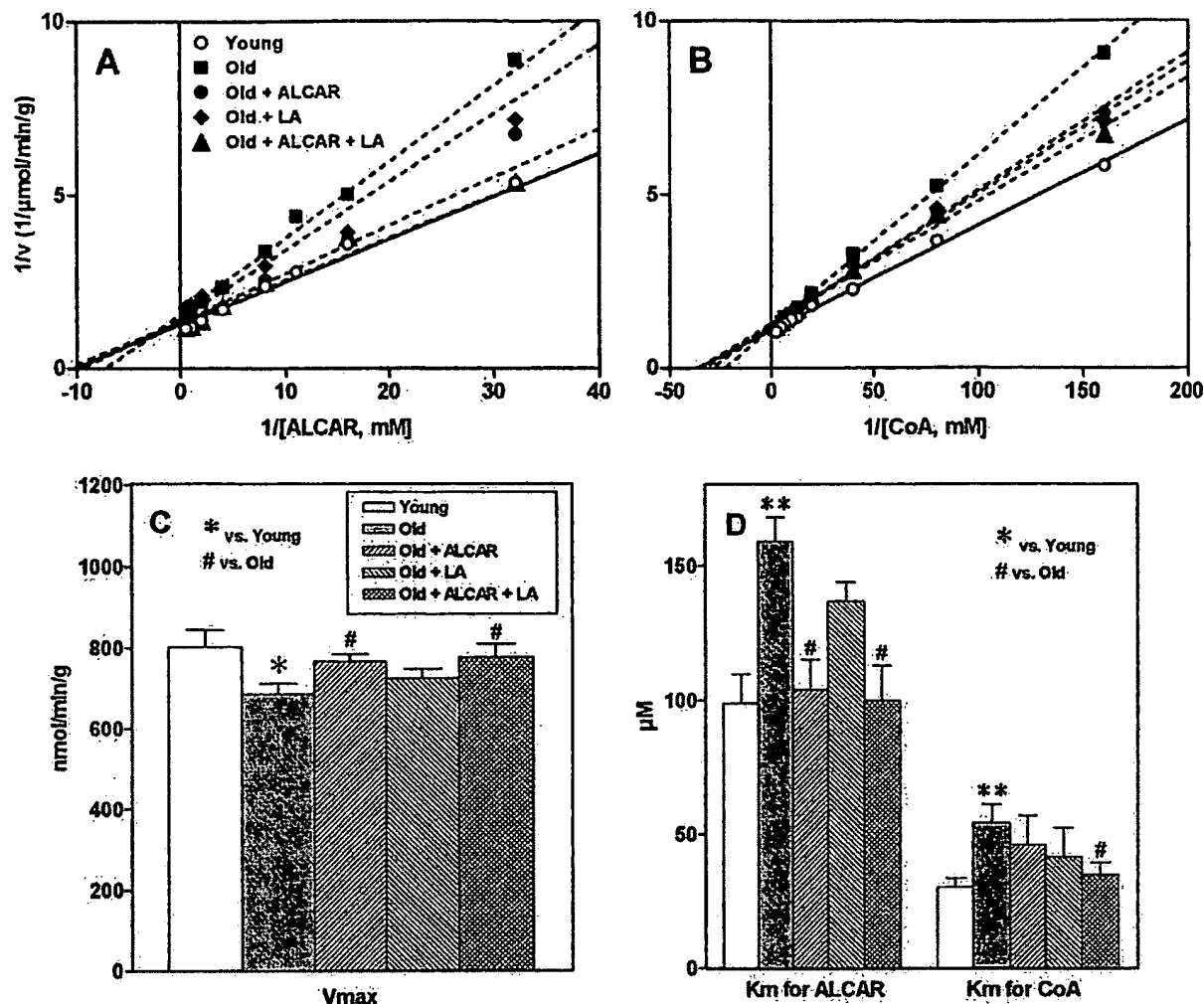


Fig. 1. Double-reciprocal plots of reaction velocity versus substrate ALCAR (A) or CoA (B) concentrations in rat brain. (C) V_{max} ; (D) apparent K_m for ALCAR and CoA. All values are mean \pm SE of 10 animals for young and old groups, 5 for the LA group, and 6 for the ALCAR and ALCAR plus LA groups. Significant difference was calculated by using Student's *t* test between young and old groups (*, $P < 0.05$, **, $P < 0.01$) and by using one-way ANOVA with Dunnett's multiple comparison test between old and other treated groups (#, $P < 0.05$).

deferoxamine, at 1 mM concentration, protected the enzyme from inactivation by 70 and 94% respectively, as did the sulfhydrylantioxidants (all at 1 mM), reduced glutathione (23%), DTT (32%), and dihydrolipoic acid (35%), although they were less

effective. Catalase (1 mg/ml) also showed protection (87%), suggesting that enzyme inactivation is mediated by Fenton chemistry. The substrates of the enzyme protected against Fe(II)-induced inactivation: ALCAR at 1 mM, 92%; L-carnitine at 1 mM, 36%; CoA at 0.6 mM, 91%; and acetyl-CoA at 0.6 mM, 50%.

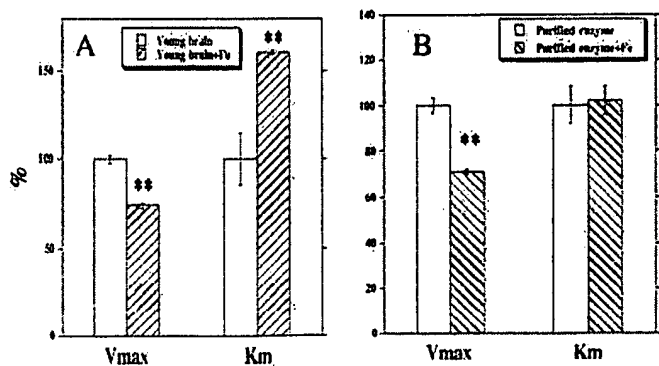


Fig. 2. The kinetic parameters (V_{max} and K_m for ALCAR) of CAT for young-rat brain with and without 5 mM Fe(II) (A) and for purified CAT (from pigeon breast muscle) with and without 0.1 mM Fe(II) (B). Significant difference was calculated with Student's *t* test (**, $P < 0.01$).

In Vivo Lipid Peroxidation Levels in Rat Brain. Compared with young rats, old rats showed a significant increase in brain MDA, a major product of lipid peroxidation. Old rats fed LA or LA plus ALCAR had significantly lowered levels of brain MDA (Fig. 3).

Effect of MDA and HNE on the K_m of Purified CAT. Fig. 4A and B show the effects of MDA and HNE in PBS on reciprocal plots of CAT for the substrate ALCAR. Both MDA and HNE caused a concentration-dependent inactivation of CAT accompanied by an increase in K_m when incubated in PBS (Fig. 4) or in TBS (data not shown). MDA was a more powerful inhibitor than HNE. In PBS incubation, MDA at 25, 50, and 100 μM inhibited CAT activity to 69, 54, and 30% and increased the K_m for ALCAR to 135, 152, and 259%, whereas HNE at 0.5, 0.75, 1.0, and 2.0 mM inhibited CAT activity to 96, 88, 79, and 60% and increased the K_m for ALCAR to 135, 164, and 269%. The concentration

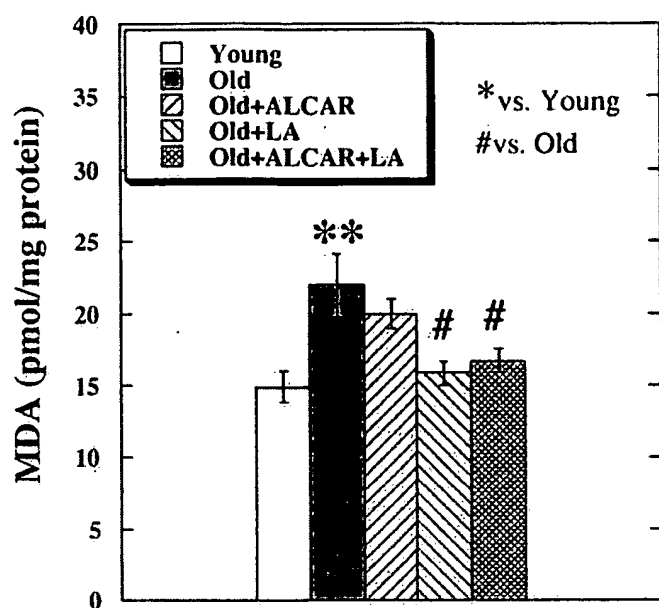


Fig. 3. MDA levels in the rat brain measured with a gas chromatography-mass spectrometric assay. The values are mean of seven animals for the young and old groups, three for the LA group, and five for the ALCAR and ALCAR plus LA groups. Significant difference was calculated by using Student's *t* test between young and old groups (**, $P < 0.01$), and by using one-way ANOVA with Dunnett's multiple comparison test between old and other treated groups (#, $P < 0.05$).

required for 50% inhibition of CAT activity by MDA is $45 \mu\text{M}$ in PBS solution and $400 \mu\text{M}$ in TBS solution. Preincubation with substrates, ALCAR, L-carnitine, CoA, and acetyl-CoA protected MDA-induced CAT inactivation and increase in K_m (Fig. 4C).

Total Metal Content in Rat Brain. Compared with young rats, old rats had a significant increase in total iron (young, 66.4 ± 1.8 , and old, 81.6 ± 2.2 ng/mg of dry tissue; $P < 0.001$) and copper (young, 10.3 ± 0.2 , and old, 17.4 ± 0.6 ng/mg of dry tissue; $P < 0.001$) in the brain; no changes in Ca, Mg, Zn, and Mn were found (data not shown). Supplementing with ALCAR and/or LA did not cause a significant decrease in the levels of total iron (old + ALCAR, 85.1 ± 4.2 ; old + LA, 80 ± 1.7 ; and old + ALCAR + LA, 76.3 ± 4.5 ng/mg of dry tissue) or total copper (data not shown).

Discussion

Old-rat brain is shown to have a moderate age-associated decrease in CAT activity and a marked decrease in binding affinity for the substrates ALCAR (young $100 \mu\text{M}$, old $150 \mu\text{M}$; Fig. 1D) and CoA. Feeding old rats ALCAR for 7 weeks, which elevates the level of free and acyl carnitines in blood and brain to a level of about $100 \mu\text{M}$ (20), significantly restored this age-associated decrease in binding affinity for ALCAR; the combination of ALCAR and LA significantly restored both CAT activity and its binding affinity for the substrates ALCAR and CoA to the levels observed in young rats. CAT has two separate binding sites: one for CoA/acetyl-CoA involving the sulfhydryl group of a cysteine residue and a second for L-carnitine/ALCAR (56). Feeding old rats LA significantly enhanced the effect of ALCAR, although LA alone had only a small effect on CAT activity and substrate-binding affinity.

Although extrapolation from *in vitro* to *in vivo* results should be viewed with caution, we suggest two plausible mechanisms that could account for the age-associated loss of binding affinity

and activity: (i) adduction to the protein of aldehyde products of lipid peroxidation, or (ii) oxidation of the protein either directly by oxidants or by metal-catalyzed oxidation. We propose that the adduction mechanism is more likely. *In vivo*, brain MDA, derived from lipid peroxidation, increases with age in parallel with a decrease in CAT activity and binding affinity for substrates (Fig. 4A). We also show that MDA and HNE, another lipid peroxidation product, decrease the V_{max} and binding affinity of CAT *in vitro*, whereas a direct oxidant, i.e., iron, does not. Lipid peroxidation may be due in part to age-associated increases in iron and copper levels. In agreement with this are our results from the *ex vivo* oxidation of young-rat brain with Fe(II), in which CAT K_m and activity change in the same way as during aging. Aldehyde products from lipid peroxidation of membranes have been shown to react with both amino and sulfhydryl groups in protein (57), thus potentially inactivating them (53, 58). The level of MDA needed to inhibit CAT *in vitro* is consistent with the MDA level observed *in vivo*. The level of MDA required for 50% inhibition of CAT is $45 \mu\text{M}$, which is not too far from its concentration in brain (20 pmol/mg of protein, i.e., about $4 \mu\text{M}$ in tissue) (Fig. 3). The MDA concentration in mitochondria is likely to be much higher. A fraction of MDA is bound to proteins (59). Most *in vitro* studies used a $10\text{--}10,000 \mu\text{M}$ range of MDA to show it toxic or mutagenic (57). MDA and HNE are only two of the many known active aldehydes formed from lipid peroxidation, many of which may contribute to enzyme inactivation.

Lowering aldehydes from lipid peroxidation does not seem to be the sole explanation for the effects of ALCAR and LA on improving CAT function and K_m . Although the combination of ALCAR and LA lowered MDA levels and restored CAT function, the results with the individual compounds indicate a more complex model. *In vivo*, LA significantly lowered MDA levels, whereas ALCAR did not, yet ALCAR significantly restored CAT function, whereas LA did not. Extrapolating from *in vitro* experiments to *in vivo* conclusions, however, depends on physiological concentration and time, as both mitochondria and protein turn over, and a definitive conclusion as to mechanism is not yet possible. *In vitro* enzyme inactivation by aldehydes and the protective effect of the substrate ALCAR (Fig. 4C) are likely to explain the *in vivo* decrease with age of CAT-binding affinity and V_{max} and their reversal by feeding ALCAR. Then why does LA not appreciably improve CAT function by itself, although it lowers the level of aldehydes? Our data show that LA enhanced the effect of ALCAR on K_m , especially for CoA, therefore LA may increase binding affinity and enzyme function to a small extent. LA is synergistic or additive with ALCAR in a number of studies (Figs. 1 and 2). Thus, the most likely mechanism for our observations appears to be the interaction of aldehydes from lipid peroxidation with CAT and a protective effect of the substrate ALCAR, with the additional beneficial effect of LA's contribution in lowering mitochondrial lipid peroxidation.

The observed improvement of CAT activity and binding affinity by ALCAR and LA may depend on protein and mitochondrial turnover. Damaged proteins and mitochondria are turned over by proteasomal and lysosomal degradation, respectively (60). Oxidative damage, especially lipid peroxidation, may be responsible for some forms of proteasome dysfunction in the central nervous system, by blocking either substrate binding or protein modification (61). Inactivation of key metabolic enzymes by mixed-function oxidation reaction has been suggested in protein turnover and aging (62). Enzymes with aldehyde-inactivated SH groups can be reactivated by excess reduced glutathione and cysteine (57). It is possible LA may play a role in reactivating CAT and in preventing proteasomes from oxidative modification.

MDA was more potent than HNE in affecting CAT kinetics. This may be because: (i) 4-hydroxyalkenals are highly specific reagents for SH groups, although they may also modify lysine,

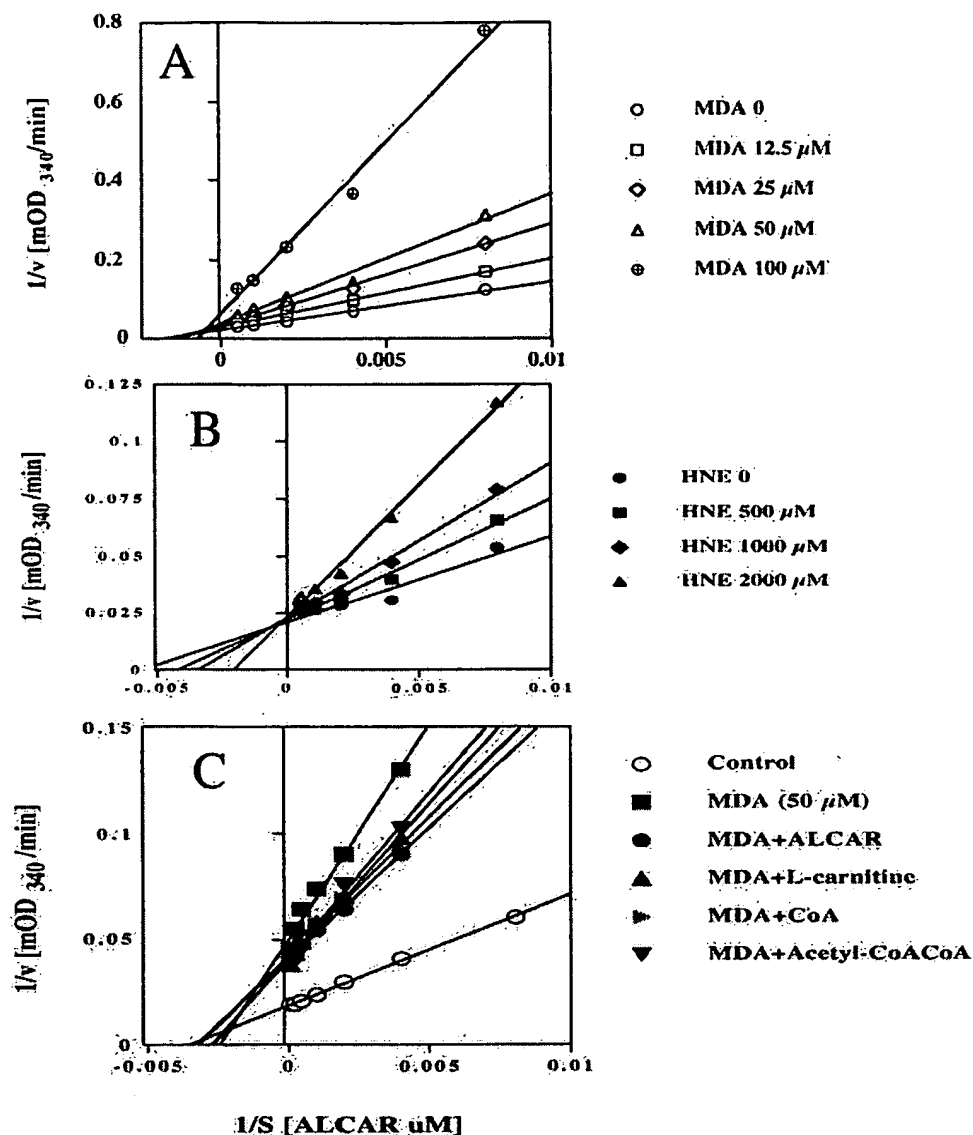


Fig. 4. Concentration-dependent effects of MDA (A) and HNE (B) in PBS on reciprocal plots of CAT for substrate ALCAR. Different concentrations of MDA or HNE were incubated with 0.36 $\mu\text{g}/\text{ml}$ of CAT enzyme for 1 h at room temperature in PBS. The kinetics were assayed with an assay mixture containing 0.036 $\mu\text{g}/\text{ml}$ of CAT. (C). The protection of substrate ALCAR, L-carnitine, CoA, and acetyl-CoA on reciprocal plots of CAT for substrate ALCAR (0.5 mM), L-carnitine (0.25 mM), CoA (25 μM), and acetyl-CoA (50 μM). The MDA used was 50 μM with 1-h incubation. The substrates were added before MDA.

histidine, serine, and tyrosine; and (ii) MDA can readily modify proteins under physiological conditions, although it is less reactive with free amino acids. MDA reacts primarily with lysine residues and can then form more stable intra- and intermolecular crosslinks (57). The effect of MDA on the activity and K_m of CAT was reduced greatly in TBS, presumably due to the relative stable covalent binding of MDA to the amino group of Tris. The effect of HNE, unlike that of MDA, on the activity and K_m of CAT was not greatly affected by TBS. In experiments with malondialdehyde, there are two possible complications whose importance has not yet been clarified: (i) when MDA is prepared from bis-acetal, small amounts of β -ethoxy or β -methoxy acrolein, highly reactive aldehydes, are unavoidably formed during acid hydrolysis (63), and a variety of similar 2-alkenals are formed during lipid peroxidation including HNE; and (ii) MDA in solution forms reactive aldol type condensation products including dimers and trimers (64), and these condensation products may also modify proteins (57).

The enzyme dysfunction induced by lipid peroxidation products such as MDA and HNE, rather than being specific for CAT, may be a common mechanism of age-associated dysfunction of enzymes with amino and sulfhydryl groups at or near their active sites. We have shown that HNE also causes a decrease in pyruvate dehydrogenase (PDH)-binding affinity for pyruvate (data not shown), confirming a study on the loss of activity of PDH by HNE (58). MDA also causes a loss of PDH activity and a decrease in binding affinity for pyruvate (data not shown).

The brain tissue of old rats showed a significant increase in iron and copper accumulation, which can cause oxidative damage by catalyzing oxidant generation and lipid peroxidation. It should be emphasized, however, that we have assayed total iron, not free redox active iron. MDA accumulates with age (Fig. 3B) in parallel to the increase in iron and copper (see *Results*). *Ex vivo* oxidation of young-rat brain with Fe(II) induced similar reduction of enzyme activity and binding affinity; *in vitro* oxidation of purified CAT with Fe(II) inactivated the enzyme but did not

alter the binding affinity. This increase of iron is consistent with previous studies in liver and brain using the atomic absorption technique (J.L., J.-Y. Park, Q. Jiang, L. Youngman, H. Atamna, and B.N.A., unpublished work) and spectrophotometric measurements (65). Although ALCAR and/or LA did not significantly effect transition metal accumulation in these short-term studies, the possibility of chelating the labile or "free" transition metals in the brain and consequently inhibiting oxidative damage cannot be excluded, as the accumulation of metals and oxidative damage is a lifelong process. LA, in addition to its oxidant-scavenging effect, is an efficient chelator of copper (66) and iron (67) that reduces the catalytic activity of transition metals in oxidant generation reactions. A higher dose of LA (0.5%) did in fact reduce iron in old-rat brain (J. H. Suh and T. M. Hagen, personal communication). Although iron and copper accumulation with age remains plausible as a cause of the increased lipid peroxides, further studies are warranted.

This study, as well as others on the effects of ALCAR and/or LA on cognition (33) and mitochondrial functions (5, 6, 68), and studies (B.N.A., J.L., and I. Elson-Schwab, unpublished work) of the age-associated decrease in binding affinity of other brain- and memory-related enzymes and receptors suggest that a decrease in enzyme-binding affinity by oxidative damage is an important contributor to age-associated memory decline, which may be ameliorated by feeding high doses of mitochondrial enzyme substrates and antioxidants.

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Memory loss in old rats is associated with brain mitochondrial decay and RNA/DNA oxidation: Partial reversal by feeding acetyl-L-carnitine and/or *R*- α -lipoic acid

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Accumulation of oxidative damage to mitochondria, protein, and nucleic acid in the brain may lead to neuronal and cognitive dysfunction. The effects on cognitive function, brain mitochondrial structure, and biomarkers of oxidative damage were studied after feeding old rats two mitochondrial metabolites, acetyl-L-carnitine (ALCAR) [0.5% or 0.2% (wt/vol) in drinking water], and/or *R*- α -lipoic acid (LA) [0.2% or 0.1% (wt/wt) in diet]. Spatial memory was assessed by using the Morris water maze; temporal memory was tested by using the peak procedure (a time-discrimination procedure). Dietary supplementation with ALCAR and/or LA improved memory, the combination being the most effective for two different tests of spatial memory ($P < 0.05$; $P < 0.01$) and for temporal memory ($P < 0.05$). Immunohistochemical analysis showed that oxidative damage to nucleic acids (8-hydroxyguanosine and 8-hydroxy-2'-deoxyguanosine) increased with age in the hippocampus, a region important for memory. Oxidative damage to nucleic acids occurred predominantly in RNA. Dietary administration of ALCAR and/or LA significantly reduced the extent of oxidized RNA, the combination being the most effective. Electron microscopic studies in the hippocampus showed that ALCAR and/or LA reversed age-associated mitochondrial structural decay. These results suggest that feeding ALCAR and LA to old rats improves performance on memory tasks by lowering oxidative damage and improving mitochondrial function.

Memory, i.e., performance on memory tasks, declines with age in animals. In the case of age-related human neurodegenerative diseases, such as Alzheimer's disease (AD), the deficit can be severe (1–4). Memory loss is accompanied but not necessarily caused by accumulation of oxidative damage to lipids, proteins, and nucleic acids, and by mitochondrial decay, all of which can disrupt neuronal function (5–10).

R- α -lipoic acid (LA) is a coenzyme that is involved in carbohydrate utilization necessary for the production of ATP in mitochondria; it is reduced in mitochondria to dihydrolipoic acid (DHLA), a potent antioxidant (11, 12). LA improves long-term memory in aged female NMRI mice (13).

L-Carnitine is a betaine required for the transport of long-chain fatty acids into the mitochondria for β -oxidation, ATP production, and for the removal of excess short- and medium-chain fatty acids (14, 15). Acetyl-L-carnitine (ALCAR) is more widely used than L-carnitine in animal and clinical studies because it enters cells and crosses the blood–brain barrier more efficiently (16). ALCAR improves cognitive function and neuronal bioenergetic mechanisms in rats with both acute and long-term treatments (17–23).

Several clinical studies report the beneficial effects of ALCAR or LA: ALCAR administration in a small group of patients with AD that resulted in improved spatial orientation and short-term memory (24, 25). LA administration in patients with AD for

approximately 1 year also resulted in mild cognitive improvements and stabilization of global neuropsychological test scores (26). Thus, as both ALCAR and LA improve mitochondrial decay, their combination may be complementary in decreasing oxidative damage to neurons and cognitive dysfunction.

As our understanding of the importance of mitochondrial decay in aging advances (27–29), the importance of improving mitochondrial function by dietary interventions of mitochondrial metabolites such as ALCAR or LA becomes clearer (30–33). Feeding 0.15–0.5% ALCAR to old rats elevated the levels of carnitine in plasma and brain to that of young rats (34) and 0.1–0.2% LA (T.M.H., unpublished data) was as effective in improving mitochondrial function in the liver as the higher doses originally used (30–33). We have examined the effects of these lower doses of ALCAR, LA, and their combination on spatial memory by using the Morris water maze, on temporal memory by using the peak procedure, decay in mitochondrial structure in the hippocampus, and oxidative damage to nucleic acids in the hippocampus and cortex.

Materials and Methods

Materials. ALCAR (hydrochloride salt) was a gift of Sigma Tau (Pomezia, Italy), and LA was a gift of Asta Medica (Frankfurt/Main, Germany). All other chemicals were reagent grade or the highest quality available from Sigma.

Animals and Diet. Fischer 344 male rats were obtained from the National Institute on Aging. Control animals were fed AIN93M diet from Dyets (Bethlehem, PA) and MilliQ water (pH 5.2). The rats in the experimental groups were fed either 0.5% or 0.2% (wt/vol) ALCAR in MilliQ water (pH was adjusted to 5.2 with 1 N NaOH), 0.2% or 0.1% (wt/wt) LA in AIN93M diet, or a combination of (0.5% ALCAR and 0.2% LA) or (0.2% ALCAR and 0.1% LA). The food consumption was determined by weighing the diet and measuring the volume of water weekly; the average daily consumption was then calculated. The weight gain during the course of the experiment was also measured. We did not find any significant differences in diet, water consumption, or weight gain between the unsupplemented old rats (13.4 ± 0.5 g/day; 18.6 ± 1.19 ml/day; body weight from 416.1 ± 14.4 to 409.2 ± 10.1 g mean \pm SE) and the old supplemented rats (For example, the ALCAR + LA group 13.1 ± 0.4 g/day; 18.4 ± 0.9 ml/day; body weight from 416.0 ± 19.0 to 414.9 ± 9.4 g; mean \pm SE). All animals were

Abbreviations: ALCAR, acetyl-L-carnitine; LA, *R*- α -lipoic acid; oxo8dG, 8-hydroxy-2'-deoxyguanosine; oxo8G, 8-hydroxyguanosine; AD, Alzheimer's disease.

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acclimatized at the Northwest Animal Facilities on the University of California at Berkeley campus for at least 2 weeks before treatment. Rats were housed individually and provided with ALCAR and/or LA for 7 weeks. The young and old rats were 4.5 and 24.5 months old at the start of the experiment; they were more than 7 weeks older at the time of death. Death, by approved protocol, was with an overdose of ether.

Morris Water Maze Test of Spatial Memory. The Morris water maze task tests spatial memory by requiring rats to find a submerged platform in a pool of water using external visual cues (35, 36). The time required for individual rats to find the platform and the length of the swim path was measured by using a digital camera and a computer system to record movement (VideoMex-V, Columbus Instruments, Columbus, OH). Trials (4 consecutive days, 4 trials per day) were with the same hidden platform location, but with varied start locations. On day 5, the platform was removed from the pool (transfer trial, 60 sec), and the time spent at the actual site where the platform was located was examined. On day 6, the time required to reach a visible platform was measured to determine visual function and motor ability.

Peak Procedure Test of Temporal Memory. The peak procedure is a modified fixed-interval schedule commonly used to study temporal memory (37). Rats were tested in 18 identical boxes that contained a light source and a speaker (for delivering light or noise signals) and a lever that dispenses single food pellets (45 mg) when pressed (mix T101, Bioserv, Frenchtown, NJ). The food supply of the rats was decreased to 85% of the free-feeding amount. In this test, the animal is rewarded with one pellet only if the lever is pressed at 40 sec from the signal. In 20% of the tests, no food was given, and an empty trial and the signal lasted 195 sec plus a geometrically distributed duration that averaged 50 sec. The results are presented as a sum of the two types of tests. Peak rate, which is the maximum response rate in a given trial and reflects the rats' choices of what responses to make and their motivation, was measured.

Electron Microscopic Observations. A subset of rats from each experimental condition was perfused transcardially with 2.5% glutaraldehyde for 2 h. The brain was removed from the skull and the hippocampus was postfixed in 0.1 M PBS with 1% osmium tetroxide. The tissues were block-stained with uranyl acetate and embedded in Epon. Sections were cut at 0.6–0.9- μ m thick from the block, stained with uranyl acetate and lead citrate, and examined with a JEOL 100 CX electron microscope.

Immunohistochemical Studies. A subset of rats from each treatment condition was anesthetized with ether and perfused with 4% paraformaldehyde for 1.5–2 h. The brain was removed and postfixed for preparing paraffin sections. Sections of hippocampus were incubated with monoclonal anti-8-hydroxy-2'-deoxyguanosine/8-hydroxyguanosine (oxo8dG/oxo8G; 1:2000; QED Bioscience, San Diego) and visualized by using standard immunocytochemical methods. Two independent analyses were done on each rat. To determine whether DNA or RNA was oxidatively damaged, sections were pretreated with either 10 units/ μ l of RNase-free DNase I or 10 mg/ml of DNase-free RNase (Roche Molecular Biochemicals) for 3 h prior to incubation with oxo8dG/oxo8G Ab (38). To quantify the extent of oxo8G/oxo8dG immunolabeling, a 525 \times 410 μ m area of staining was captured by using a \times 2.5 photo eyepiece, a Sony (Tokyo) high-resolution charge-coupled device (CCD) video camera (XC-77), and the built-in video capture capabilities of a Macintosh 8100/80AV. All sections from a given region were captured sequentially during one session and were analyzed blind with respect to treatment condition. Subsequently, public domain image analysis software (IMAGE 1.55, National Institutes of

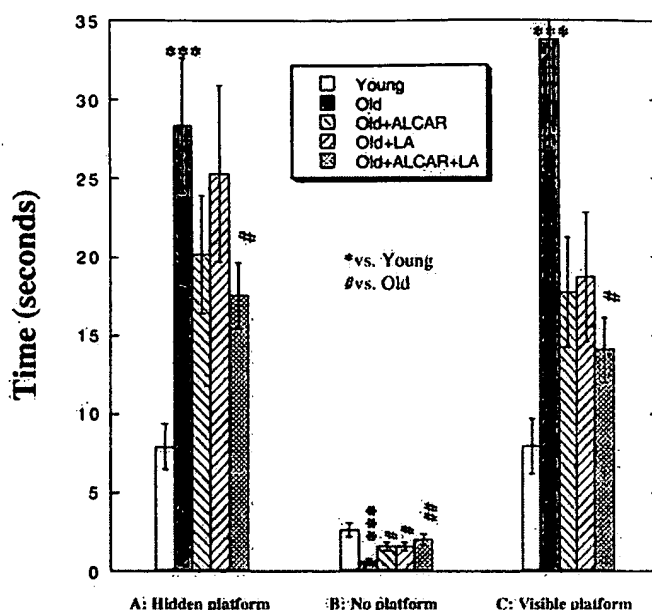


Fig. 1. Morris water maze test in relation to age and treatment. (A) Time on day 4 taken to find the hidden platform. (B) Time spent at the former platform position in the transfer test. (C) Time to find the visible platform. Data are mean \pm SEM of 9 rats in young and old, 5 in LA (0.1%), and 6 in ALCAR (0.2%) and ALCAR + LA groups. Higher doses, 0.2% LA and/or 0.5% ALCAR, showed similar results (data not shown). Statistical differences were examined with two-tailed Student *t* test. ***, $P < 0.001$ vs. young rats; #, $P < 0.05$ and ##, $P < 0.01$ vs. old rats.

Health) and gray-scale thresholding were used to separate positive staining from background and to calculate the percentage of area occupied by oxo8G/oxo8dG immunoreactivity.

Results

Spatial Memory. Rats are proficient swimmers and are motivated to escape from water. Once animals learn where the hidden platform is located, they can remember the location and swim rapidly to it from any starting point. Both time taken to reach the platform (Fig. 1) and swimming distance traveled (data not shown) were measured and gave similar results. Fig. 1A shows results obtained on day 4. Young rats spent a significantly shorter time than old rats ($P < 0.001$) in finding the hidden platform. ALCAR or LA seems to shorten the time in old rats, but the differences were not significant. However, the combination resulted in significantly shorter times ($P < 0.05$) as compared with old control rats. The tracks of individual rats on successive trials and days have been shown (34).

A transfer test, in which the platform was removed, was carried out on day 5. The time spent at the previous platform position is a measure of search accuracy and spatial memory. Young rats spent significantly more time at the former platform position ($P < 0.001$) than old rats did. The ALCAR ($P < 0.05$) and LA ($P < 0.05$) significantly restored the lost procedural subcomponent of spatial memory and the combination was even more effective ($P < 0.01$; Fig. 1B).

A clearly visible platform was used to measure deficits in vision, motivation, motor strength, or coordination on day 6 of the training cycle. The platform protruded 1 cm above the surface of the water. Young rats required less time to find the visible platform than the old animals (Fig. 1C). All three supplementation groups showed improvement, but only the combination treatment group reached statistical significance (Fig. 1C).

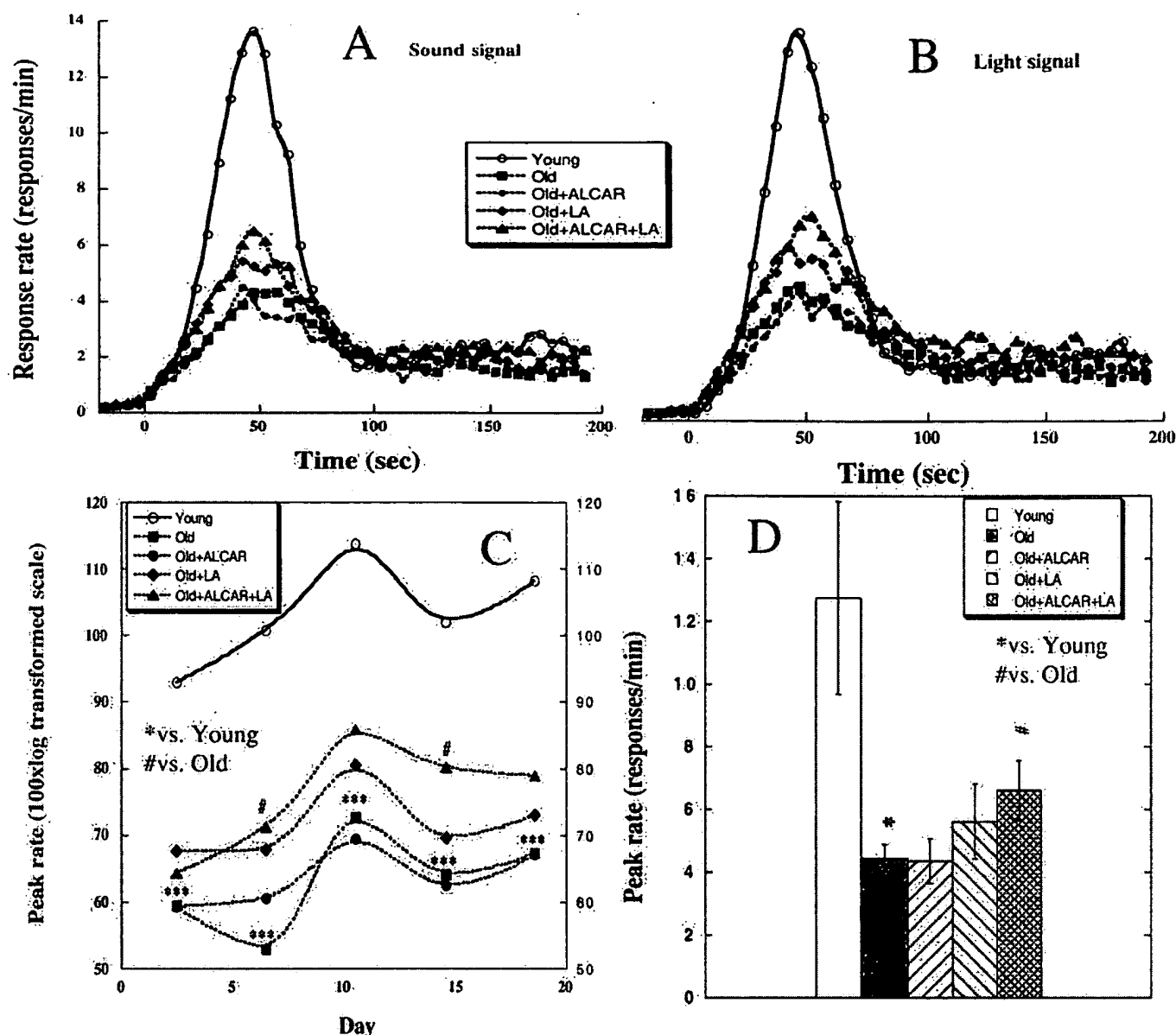


Fig. 2. Peak procedure test related to age and treatment. Response rate functions plotted separately for sound-signal trials (A) and light-signal trials (B) obtained during the last 10 days of the test. (C) Peak rate over the 20 days of peak procedure testing. Each data point averages 2 days of testing. (D) Peak rate of the last 10 days averaged. Data are mean \pm SEM of 6 in young, 7 in old, 4 in LA (0.2%), and 5 in ALCAR (0.5%) and ALCAR + LA groups. Treatment with lower doses, 0.1% LA and/or 0.2% ALCAR, showed similar results (data not shown). Statistical differences were examined with two-tailed Student *t* test. *, *P* < 0.05 and ***, *P* < 0.001 vs. young rats; #, *P* < 0.05 vs. old unsupplemented rats.

Temporal Memory. The response rate to a sound (Fig. 2A) and to a light (Fig. 2B) signal is the same, indicating that the rats responded similarly to both signals. Results from the last 10 days of testing were used, where responses had reached asymptotic levels.

Peak rate (Fig. 2C and D) of young animals was significantly higher than that of all other groups: young compared with old (*P* = 0.001); young compared with old + ALCAR (*P* = 0.004); young compared with old + LA (*P* = 0.043); and young compared with old + ALCAR + LA (*P* = 0.046). Although ALCAR does not show any significant effect (comparing the old + ALCAR group to the old control rats), LA seems to slightly increase peak rate. The old + ALCAR + LA treatment showed a more significant increase (*P* = 0.033) in peak rate in old animals than treatment with LA alone.

Ultrastructural Observations of Neuronal Mitochondria. Electron microscope observations of hippocampal neuronal mitochondria indicate that structural abnormalities develop with age. Compared with young rats, old rats showed some disruption and loss of cristae in about half of the mitochondria in the dentate gyrus area, indicating structural decay. Animals treated with 0.5% ALCAR and/or 0.2% LA showed less structural disruption and loss of cristae. In addition, old rats had more lipofuscin in the cytoplasm of granule cells of the dentate gyrus, and the combined treatment rats also seemed to have less lipofuscin. However, these results were obtained from one or two animals per group. Clearly, further quantitative studies with more animals and more fields are needed to confirm these observations.

Oxidative Damage to Nucleic Acids. Various regions of the brain were stained with an Ab that recognizes oxidized DNA or RNA

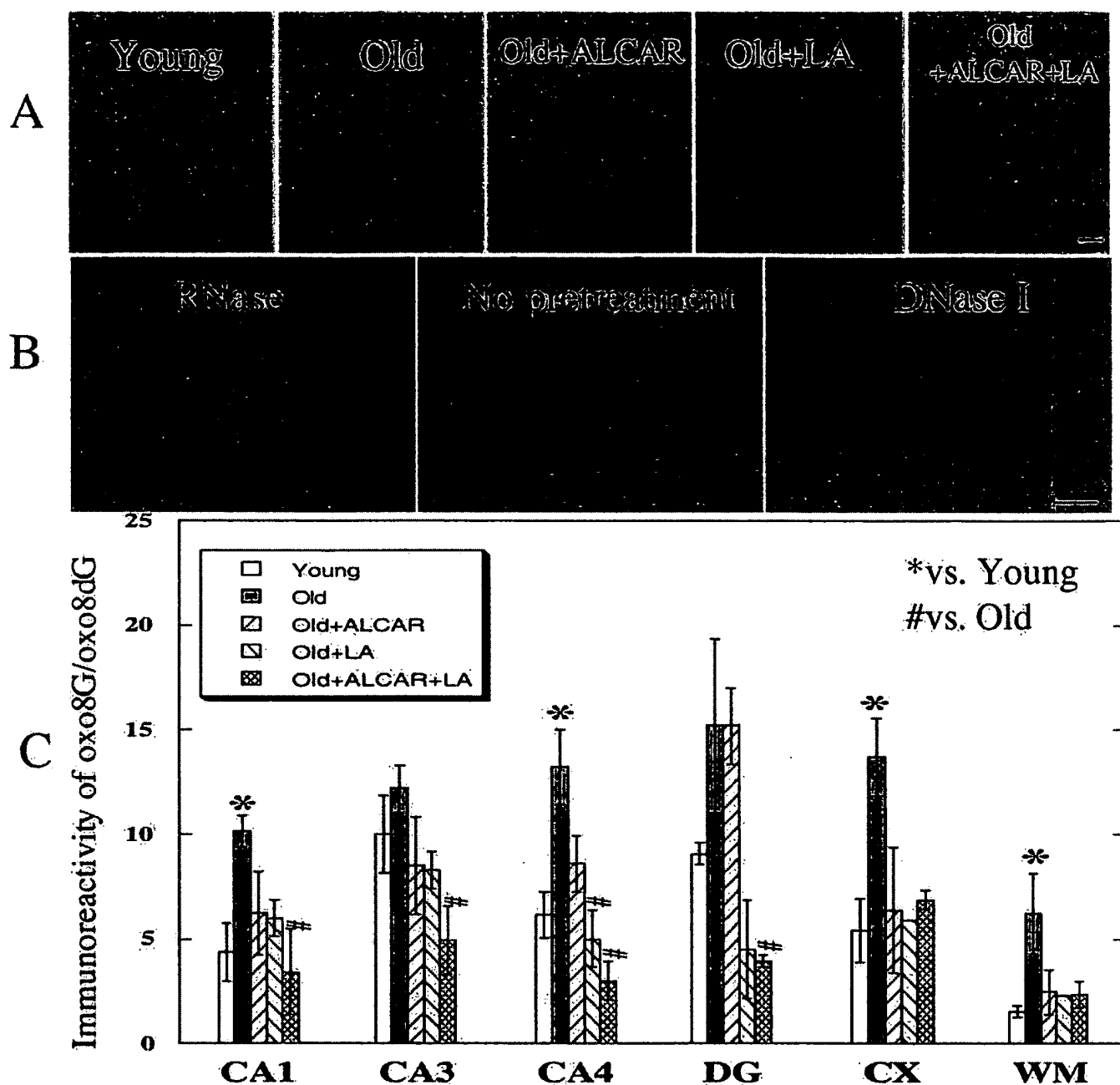


Fig. 3. Immunostaining relative to age and treatment for oxidized nucleic acids in neurons. (A) Representative photographs of oxo8G immunoreactivity in area CA1 of the hippocampus and adjacent white matter, from individual rats selected from young, old, old + ALCAR (0.5%), old + LA (0.2%), and old + ALCAR (0.5%) + LA (0.2%) groups. (B) CA1 sections pretreated with either DNase or RNase before incubation with Ab. (C) Extent of immunoreactivity to oxo8G in the hippocampus [CA1, CA3, CA4, dentate gyrus (DG), cerebral cortex (CX), and white matter (WM) in rat brain]. [Bar = 50 μ m.] Values are mean \pm SEM of 5 animals for young and old groups, 3 for old + ALCAR and old + LA groups, and 2 for the old + ALCAR + LA group. The Mann-Whitney *U* test was used to compare values. *, $P < 0.005$ vs. young rats; #, $P < 0.05$ vs. old control rats.

(oxo8dG or oxo8G; ref. 39). Fig. 3A shows representative images of the CA1 region of the hippocampus. Fig. 3C shows that old rats without treatment showed significantly higher immunoreactivity than young rats in areas CA1, CA4, cerebral cortex, and in the white matter. Both ALCAR and LA reduced immunoreactivity, but only LA showed a significant effect in the CA4 region. The combination showed a significant effect on lowering immunoreactivity in CA1, CA3, CA4, and dentate granule cells in old rats.

Fig. 3B illustrates that pretreatment of sections, including area CA1 with RNase but not DNase, virtually eliminated the im-

munoreactivity, indicating that the predominant damage to neuronal nucleic acids is to RNA (oxo8G). In CA1, RNase pretreatment reduced the immunoreactivity by 92%, whereas DNase, rather than reducing, enhanced (168%) the immunoreactivity (Fig. 3B).

Discussion

Old rats have increased mitochondrial dysfunction and oxidative damage, which is associated with cognitive deficits in both spatial and temporal memory. Spatial memory relies on intact hippocampal function. Temporal memory may also be associated

with the hippocampus, although it may be more closely associated with the striatum and cerebellum. The dietary administration of a combination of ALCAR and LA to old rats improves mitochondrial function in liver (40). The purpose of this study was to determine whether it also improves cognition.

Spatial memory was assayed in the Morris water maze. The Morris water maze has been used extensively to measure cognitive deficits in spatial memory in lesion studies (41–47) and in aging (48–52). Old rats showed decreased spatial memory compared with young rats; ALCAR and/or LA restored some of this function, the combination being more effective than each compound alone. We also observed significant age effects in the transfer test, which measures search accuracy and is considered a procedural (habitual) subcomponent of spatial memory (36). ALCAR and/or LA significantly restored performance in this test, the combination being more effective ($P < 0.01$) and not significantly different from that of young rats (Fig. 1*B*). Schenk and Morris (36) have shown that after a retrohippocampal lesion, the procedural component of spatial memory can be partially recovered after training. We also observed significant age effects on the latencies of animals in finding a visible platform, which is a control procedure used to detect sensory motor deficits or motivational differences that impair water maze performance. The dietary interventions have similar effects on the visible platform test as those observed during the hidden platform tests (Fig. 1*C*).

Many physiological changes occur with age and can have major consequences on cognitive performance (53). We observed age and treatment effects on several noncognitive factors, such as motivation and locomotor activity, which can potentially contribute to the cognitive results. The age-associated decline in the Morris water maze test, therefore, should not be considered solely a test of cognition, but also as revealing a general decline in other systems as a result of aging. Old animals are known to be less sensitive to pain and possibly to temperature, which may affect their motivation to find the hidden platform. ALCAR and LA reduce mitochondrial dysfunction in peripheral systems (31, 33, 54, 55), including sensory systems such as hearing (56). Therefore, improvements shown here in test performances attributable to ALCAR, LA, or their combination, including the visible platform test and ambulatory activity (see ref. 40), suggest that reversing mitochondrial decay might reverse age-associated declines in nervous, cardiovascular, visual, and auditory systems, as well as general effects on motivation and physical strength.

Temporal memory, as assayed by the peak procedure, measures the function of the internal clock, learning processes, attention, and exploratory behavior. The combination of LA with ALCAR showed a significant improvement on peak rate ($P < 0.05$). The peak procedure is a time-discrimination procedure, which resembles a discrete-trials fixed-interval schedule with catch trials; it has been used to study the timing abilities of animals (37). Several studies have shown that old rats have deficits in time perception (57–61). One advantage of the peak procedure is that it allows for comparison of performance by using different types of signals and sensory modalities. The similarity of performance with light and sound signals suggests that the deficits are the results of deficits in cognition as rats of different ages do not differ in their sensitivity to light and sound at the two levels of light and sound used in this study. Peak rate reflects changes in a response learning mechanism. Old rats had lower peak rates, suggesting that old animals have difficulty learning the relevant response. The combination of ALCAR and LA seems to have a complementary effect on improving the peak procedure performance.

Not all of the old rats tested had cognitive deficits; this resulted in a large SD and the need for larger numbers of rats to achieve statistical significance. In future experiments it would be useful to separate cognitively impaired from unimpaired old rats to

show more pronounced effects in old rats that receive treatment (23, 52).

The current study also has tested the hypothesis that cognitive improvements in response to ALCAR and/or LA interventions are linked to reductions in oxidative damage in old brain. To measure oxidative damage to nucleic acids, we used an Ab that detects both oxidized DNA and RNA (39). RNase pretreatment decreased immunoreactivity extensively, whereas DNase had a smaller effect. This result suggests that the oxidized nucleic acid in the aged rat brain is predominantly RNA, which is consistent with studies in human brains with AD (38). It is clear that more than 90% immunoreactivity is from RNA, suggesting that RNA oxidation is a significant biomarker of aging in rat brain. The mechanism of the DNase enhancement of immunoreactivity remains unclear; the digestion of DNA may have unmasked binding sites allowing greater access of the mAb to the RNA. Cytoplasmic punctate staining is consistent with either cellular RNA or mtDNA/RNA. RNA being the predominant oxidized nucleic acid is consistent with the lack of staining of nuclear DNA. The type of RNA oxidized and its subcellular localization remain to be determined, particularly with respect to mitochondria, the most likely oxidant target and the one that is improved by ALCAR and/or LA. RNA oxidation increased significantly as a function of age in rats in areas CA1 and CA4 in the hippocampus, in cortical neurons, and in white matter in the frontoparietal cortex. Feeding old rats LA significantly reduced the levels of oxidized RNA in CA4. The combination of ALCAR and LA was effective in significantly reducing oxidative RNA damage in neurons in CA1, CA3, CA4, and dentate gyrus of the hippocampus to levels not significantly different from young animals.

Poorer performance on memory tasks by old rats could involve, in part, oxidative damage to RNA, with errors in translation (62) compromising protein synthesis critical for the formation of new memories (63, 64). Although oxidative damage to RNA has been shown to be more extensive than damage to DNA in urine and plasma (39), oxidized RNA has not been a focus of interest as an oxidative damage marker for brain aging or cognition, except in some patients with AD sample studies. Neuronal RNA oxidation is a prominent feature of vulnerable neurons in AD, Down's syndrome, and Parkinson's disease, all of which are diseases associated with severe cognitive deficits (38, 65, 66). Neuronal RNA oxidation may thus contribute to memory decline and serve as a sensitive marker for intervention studies. However, oxidant-induced enzyme dysfunction is also an important contributor to neuronal decay and aging (67–69).

The improving effects on performance on memory tasks by ALCAR and/or LA on hippocampal mitochondria are supported by morphological observations. There seems to be a loss of mitochondrial cristae with age. Evidence that ALCAR reversed this loss with a dose-dependent response has been presented (34). Similar to ALCAR, LA also reduced age-dependent cristae loss in the dentate granule cells of the hippocampus. Because ALCAR alone showed a virtually complete reversal of the cristae loss, we cannot say whether the combination has an improving effect or not, but it produced at least as large a reduction as the ALCAR or LA alone.

The loss of memory with age seems to be caused in good part by oxidative mitochondrial decay in neurons. (i) The effectiveness of the mitochondrial metabolites ALCAR and LA suggests that mitochondrial decay is involved. (ii) The oxidation of RNA/DNA in neurons is likely to be mitochondrial (70). (iii) Neuronal mitochondria show structural decay with age.

The cognition-improving effect of ALCAR may also be caused in part by the donation of an acetyl group for the synthesis of the neurotransmitter acetylcholine through choline acetyltransferase and carnitine acetyltransferase (17, 71, 72). Low acetylcholine levels in certain brain regions are associated with

age-related cognitive dysfunction, including AD (73). Because of the profound effects of calorie restriction, we have compared dietary intakes carefully and have found no significant differences in food and water consumption or in body weight (see *Materials and Methods*).

In conclusion, feeding old rats ALCAR and/or LA improved performance on memory tasks, reduced brain mitochondrial structure decay, and reduced oxidative damage in the brain. The combination of ALCAR and LA showed a greater effect than ALCAR or LA alone. These results suggest that feeding a combination of mitochondrial metabolites to old animals may prevent mitochondrial decay in neurons and restore cognitive dysfunction. These results also suggest that consumption of high

levels of mitochondrial metabolites may be an efficient intervention in humans for delaying brain aging and age-associated neurodegenerative diseases.

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